Comparison of the Growth and Monoclonal Antibody Production of Suspended Mammalian Cells in Three Perfusion Systems

by

Kathy Hufford

B.S. Chemical Engineering
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Submitted to the Biological Engineering Division
In Partial Fulfillment of the Requirements for the
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Signature of Author _____
Biological Engineering Division
January 19, 2007

Certified by _____
Jean-Francois Hamel
Research Engineer and Lecturer of Chemical Engineering
Associate Industrial Liaison
Thesis Supervisor

Accepted by _____
Bevin Engelward
Associate Professor of Biological Engineering
MEBE Program Director

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ABSTRACT

The purpose of this thesis was to provide a broad survey of bioprocess options for typical drug production vehicles in the biotechnology industry. This goal was accomplished by comparing the growth and monoclonal antibody production by HPCHO Chinese hamster ovary cells and IB4 hybridoma cells in batch, fed-batch, and three perfusion systems: the stirred ceramic membrane reactor (SCMR), the alternating tangential flow (ATF) hollow fiber membrane system, and the external spin filter (ESF) system.

The batch experiments for each cell line were used as base case scenarios for the comparisons of cell growth and monoclonal antibody production. The fed-batch experiments for each cell line did not vastly improve the cell growth and monoclonal antibody production over the batch cases. The SCMR perfusion system greatly improved the cell growth and monoclonal antibody production for each cell line. The maximum viable cell concentration of the IB4 hybridoma cells in the SCMR experiment was over seven times that found in the batch experiment and the monoclonal antibody production in the SCMR experiment was over ten times that found in the batch experiment. The ATF perfusion system greatly increased the cell growth for each cell line over the batch cases, but the shear forces created by the system adversely affected the viability of the cells and the monoclonal antibody production was less than that of the SCMR experiments. The ESF perfusion system damaged the HPCHO Chinese hamster ovary cells. For the production of the IB4 hybridoma cells, the ESF system, as well as a modified ESF system, did not vastly improve the cell growth and monoclonal antibody over the base case due to cell aggregation and filter fouling.

The cell growth and productivity data, along with economic considerations, were evaluated for the purpose of recommending the best feeding strategy for each of the two cell lines studied. It was concluded that the most successful operation mode for the two cell lines studied was the SCMR perfusion system. More research is needed regarding the scale-up ramifications of the SCMR perfusion system as well as in the quantification of monoclonal antibodies, the optimization of fed-batch systems, and the use of alternative perfusion systems.

Thesis Supervisor: Jean-Francois Hamel
Title: Research Engineer and Lecturer of Chemical Engineering
Associate Industrial Liaison

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1 Introduction

1.1 Animal Cell Culture and the Production of Monoclonal Antibodies

The pharmaceutical and biotechnology industries are vibrant and rapidly growing. The current demand for biopharmaceutical products is high and is expected to significantly increase over the next few years. Across the market today, there are approximately 35 biopharmaceutical products available. An estimated 700 biotherapeutics are currently in clinical development, with nearly 200 of these products in late-stage trials (1). There are approximately 92 companies located in the United States and Europe operating Good-Manufacturing-Practice facilities to manufacture biopharmaceutical products for clinical trials and market supply using mammalian cell culture. The worldwide mammalian cell culture capacity for the manufacturing of biopharmaceuticals was estimated to be 1.7 million liters in 2004 and production capacity is expected to rise 48% over the next five years (1). This drastic increase in production capacity, however, is still insufficient to meet the growing market demand for biopharmaceutical products.

One of the most important biopharmaceutical products manufactured by the pharmaceutical and biotechnology industries is the monoclonal antibody. Therapeutic monoclonal antibodies (mAbs) have developed into a beneficial and profitable class of biopharmaceutical products. The recombinant proteins currently in development are predominantly monoclonal antibodies and there are more than 150 mAb products in studies sponsored by companies worldwide (2). The market for monoclonal antibodies is expected to increase by 30% a year after having reached sales of over $6.5 billion in 2004. By 2008, 16 new monoclonal antibody products are expected to enter the market (3). The vast majority of these monoclonal antibodies are secreted from mammalian cell lines such as Chinese Hamster Ovary (CHO) cells and hybridoma cells. Human Embryonic Kidney (HEK-293) and NSO (murine myeloma) cells are also utilized to produce monoclonal antibodies.

Current research focuses on the effort to meet this increasing market need for monoclonal antibodies by increasing the volumetric productivities of the bioreactors used

for their production. Some methods of meeting this demand include improving the design of the gene vector system of the cell line, optimizing the medium used to grow the cell line, down-regulation of apoptosis to prolong cell survival, and reducing the heterogeneity of glycoforms of the product (4). Another method of meeting the increasing market need for monoclonal antibodies involves moving away from the industry standard of the stirred tank reactor (STR) and investigating alternative bioreactor designs such as the airlift bioreactor (1). An airlift bioreactor is a bioreactor in which the cell culture is kept mixed and gassed by the introduction of a gas mixture at the base of a column-like reactor. The reactor is equipped with either a draught tube or another device by which the volume of the reactor is partitioned into a gassed and ungassed region. This experimental design generates a vertically circulating flow. The idea of utilizing novel bioreactors such as the Wave bioreactor in monoclonal antibody production has also shown some promise (5). In the Wave reactor, cells are grown in a pre-sterilized bag and agitation is accomplished by waves created by gently rocking the bag back and forth. Another way to increase volumetric productivity that shows great promise involves optimizing the feeding strategy of the production process. This method is best achieved by optimizing a fed-batch or perfusion process in the STR, but can also be accomplished by combining a fed-batch or perfusion feeding strategy with an unconventional bioreactor such as the Wave reactor (4).

1.2 Feeding Strategies for Animal Cell Culture

There are three main feeding strategies in cell culture: batch, fed-batch, and continuous feeding, also known as perfusion. In the batch mode of operation, cells are inoculated into nutrient-rich medium in the bioreactor and allowed to grow undisturbed. No additional nutrients are added during the run and cell growth continues only until the concentration of one of the nutrients becomes limiting or toxic waste products build up in the culture. The volume of the liquid in the bioreactor remains constant throughout the process because nothing is being added or removed. An illustration of batch mode is shown in Figure 1.

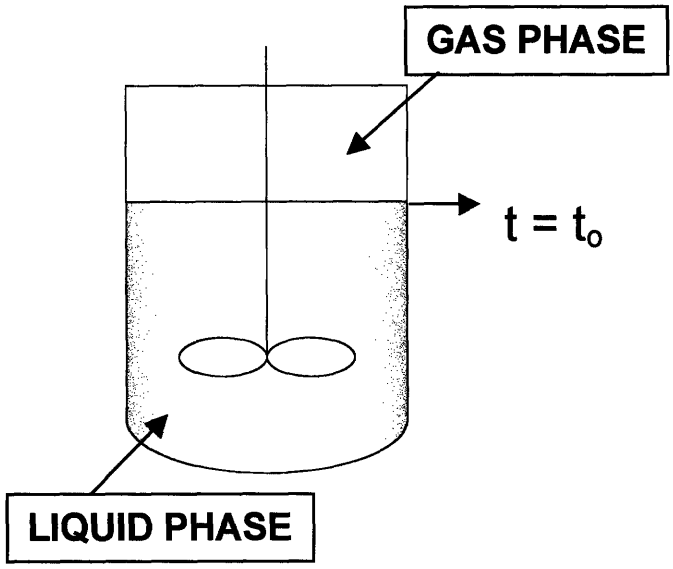

Figure 1 – A stirred tank reactor in batch mode (adapted from figure created by L. Tunney).

Another approach to stirred-tank bioreactor operation controls cell growth by the addition of nutrients at one time or several times during the cycle. This operational mode is referred to as fed-batch because the bioreactor is inoculated in the same way as in the batch process but the cell culture is fed periodically while the experiment is running. Adding nutrients to a batch culture during the run can increase the amount of product harvested (6). In this case, the volume of liquid in the bioreactor increases with time due to the addition of nutrients during the run. About 70-90% of the final working volume is inoculated into the bioreactor and the remaining 10-30% of the final working volume is added during the run at one or several different time points. An illustration of fed-batch mode is shown in Figure 2.

There are many different approaches that may be taken in designing a fed-batch process (7). Feeding may be based on the consumption of glucose or other nutrients, such as amino acids. Feeding may also be based on the production of waste products or the ratio or nutrients to waste products. In addition to determining the basis for medium addition, the medium to be added to the culture must be chosen carefully. In some cases, concentrated commercial medium is used. In other cases, an initial medium is created from stock solutions of important nutrients and then is subsequently optimized to provide the culture the best growth and production possible (8). Various issues, such as expense, ease of use, and available analytical equipment, govern the choice of feeding strategy and medium composition of the fed-batch reaction.

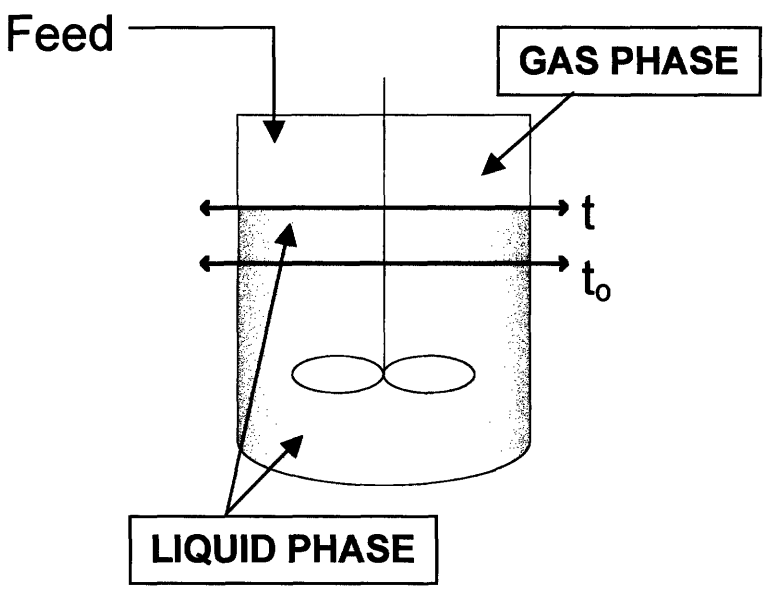

Figure 2 – A stirred tank reactor in fed-batch mode (adapted from figure created by L. Tunney).

Fed-batch operation is an excellent way to increase the volumetric productivity of a bioreactor and is becoming one of the most popular production modes in industry (6). Since fed-batch operation is a variant of the standard batch mode, cells have already been well characterized for this mode of operation. Fed-batch operation can be performed in essentially any vessel in which a batch is performed, from a stirred tank reactor to a Wave bioreactor. Because of this characteristic, it is relatively easy to change cell lines and desired products in fed-batch facilities. Fed-batch systems are usually faster, cheaper, and easier to implement and reproduce than more complex feeding strategies such as perfusion. The validation and characterization of a fed-batch process, as well as the purification of the desired product, is therefore easier than with perfusion systems. The risk of contamination is less for a fed-batch process than for a perfusion process because fed-batch experiments require less equipment and fewer disturbances to the cell culture experiment. The production levels associated with fed-batch operation are significant and some yields of up to 5 g/L have been reported (9). Although high titres have been achieved with fed-batch processes, greater volumetric productivity may be possible if growth-inhibitory by-products could be removed from the culture and a greater amount of fresh nutrients could be provided to the cells. Finally, the start-up costs and necessary plant capacities are generally higher for fed-batch processes than for perfusion processes (10).

In perfusion culture, the mammalian cells are retained in the reactor while fresh medium is added and toxic metabolites are removed. These cultures can be maintained for several weeks or months at a time. The main benefit of perfusion systems is that cells remain in the reactor while the secreted product is continuously harvested. A cell retention device is typically used to allow for the separation of waste products from the cells and this device could be external or internal to the reactor. Cell densities greater than 10^7 cells/mL and with higher productivities have been achieved with perfusion systems in much smaller reactors than in other systems (4). Some examples of methods of cell retention devices include continuous centrifuges (11), tangential flow membrane filters (12, 13), dynamic filters (14, 15), spin-filters (16, 17), ultrasonic (18, 19) and dielectrophoretic separators (15, 20), gravity settlers (15, 21), hydrocyclones (15, 22), and ceramic membrane filters (23, 24). The most important technical aspects of any perfusion system are the method of cell retention and the feed rate of fresh medium. An illustration of perfusion mode is shown in Figure 3.

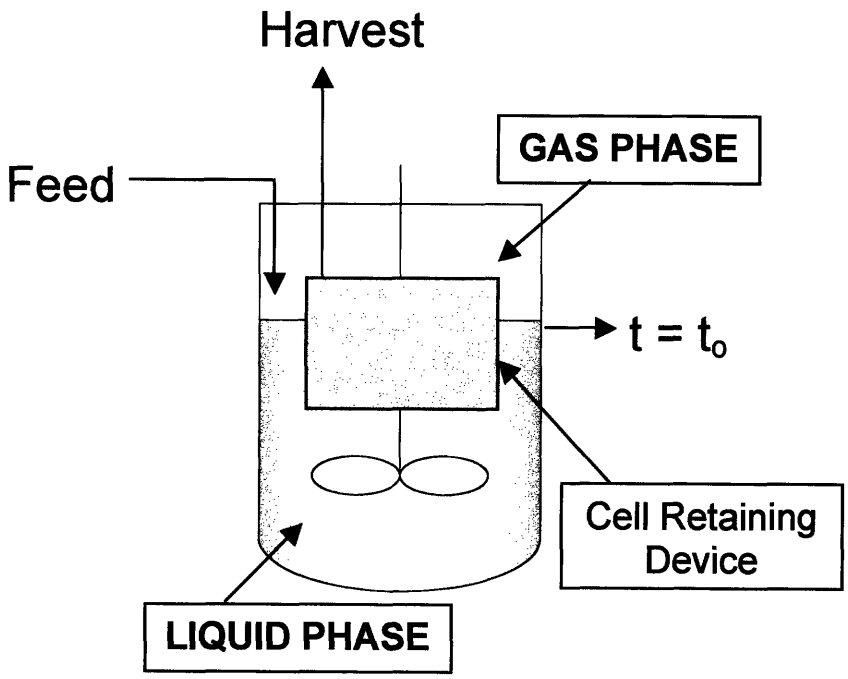

Figure 3 – A stirred tank reactor in perfusion mode (adapted from figure created by L. Tunney).

Perfusion culture of suspension mammalian cells offers several advantages over traditional batch and fed-batch modes of culture, all of which are important in the production of biological products. These advantages include higher cell densities, higher

volumetric productivities (in terms of the volume of the reactor used, rather than the medium consumed), and fast medium exchanges. A bioreactor used in a perfusion process may be up to ten times smaller for the production of the same quantity of product as would be necessary with other production modes of cell culture (4). Perfusion cultures can be more demanding to set up, can require more medium (which can be costly), may produce less consistent lots of product, and can be more susceptible to contamination than other production systems, but they provide a continuous stream of product over several weeks or months. A continuous stream of product is particularly useful if the desired product is susceptible to degradation in the culture environment or if the product stream can be transported directly to downstream processing. The economics of perfusion processes can be quite favorable in the case of specialty pharmaceutical products, since a smaller reactor and media formulated in-house can be used to create a significant amount of valuable product.

Table 1 – Direct comparison of batch, fed-batch, and perfusion strategies (adapted from 1, 6).

Process characteristics	Batch	Fed-Batch	Spin-filter / Dialysis Membrane Perfusion
Cell generations	Low	Medium	High
Cell concentration	Low	Medium	High
Volumetric productivity (reactor)	Low	Medium	High
Volumetric productivity (media)	Medium	High	Low
Throughput (product/reactor)	Low	Medium	High
Lot consistency	High	Medium	Low
Cycle time	Short	Medium	Long
Turnaround time	Low	Medium	High
Waste generation	Low	Low	High
Required materials	Low	Medium	High
Operation	Simple	Simple	More complex
Failure risk	$\leq 5\%$	$\leq 5\%$	$\leq 10\%$
Process development	Low	Medium	High
Scale-up	Easy	Easy	More complex

A direct comparison of batch, fed-batch, and various perfusion strategies is shown above in Table 1. Although the fed-batch strategy is currently the most popular operation mode in industry, there is much debate about the benefits of perfusion. Research is

underway to determine if the benefits of perfusion over fed-batch strategies outweigh the associated costs and risks associated with such complex systems.

2 Research Objective and Experimental Design

2.1 Objective of Research

The purpose of this thesis was to optimize and compare the growth and production of monoclonal antibodies (mAb) by HPCHO Chinese hamster ovary (CHO) cells and IB4 hybridoma cells in three perfusion systems: the stirred ceramic membrane reactor (SCMR), the alternating tangential flow (ATF) hollow fiber membrane system, and the external spin filter (ESF) system. The growth and productivity data gathered from the perfusion systems were also compared to growth and productivity data gathered from batch and fed-batch systems. The cell growth and productivity data were considered along with economic factors in recommending the best feeding strategy for each of the two cell lines evaluated.

The specific goals of this thesis project are outlined below:

1. To gain proficiency in operating laboratory bench scale bioreactors in batch, fed-batch, and perfusion modes.
2. To analyze cell growth and health via the automated trypan blue assay.
3. To analyze metabolite and waste concentrations of the cell culture.
4. To analyze monoclonal antibody production of the suspended mammalian cells via capillary electrophoresis (CE).
5. To experiment with different fed-batch mediums and timelines to find the fed-batch feeding strategy yielding the greatest cell growth and productivity of two suspended mammalian cell lines.
6. To optimize the SCMR system in order to find the best conditions for cell growth and mAb productivity of two suspended mammalian cell lines.
7. To optimize the ATF system in order to find the best conditions for cell growth and mAb productivity of two suspended mammalian cell lines.
8. To optimize the external spin filter system in order to find the best conditions for cell growth and mAb productivity of two suspended mammalian cell lines.

9. To compare the three systems studied in terms of cell growth, mAb production, and economic factors and recommend the best feeding strategy for two suspended mammalian cell lines.

2.2 Hypothesis

It was believed that all three perfusion systems, as well as the fed-batch system, would support cell growth to a greater maximum cell concentration and achieve a greater monoclonal antibody production than the corresponding batch system for both the HPCHO Chinese hamster ovary cells and the IB4 hybridoma cells. The fed-batch system would exhibit greater productivity per cell, but a lower maximum cell concentration than the three perfusion systems. All three perfusion systems would show productivities per cell that were greater than the batch system but less than the fed-batch system. The total productivities would nonetheless be higher in the three perfusion systems than both the batch and fed-batch systems.

From the findings of previous research (24), it was believed that the stirred ceramic membrane reactor system would reach a maximum cell concentration of IB4 hybridoma cells of approximately 3×10^7 cells/mL at a viability of greater than 90%. The productivity of this system was expected to be on the order of hundreds of milligrams per liter. Because the HPCHO Chinese hamster ovary cells reached a lower maximum cell density and showed lower productivity in batch culture than the IB4 hybridoma cells, the stirred ceramic membrane reactor system was expected to perform worse for this cell line. The alternating tangential flow hollow fiber membrane system was expected to perform worse than the stirred ceramic membrane reactor system because the shear forces resulting from the ATF pump were expected to affect the viability of the cells and limit the possible maximum cell densities and productivities. Fouling of the hollow fiber membrane was also expected after several days of perfusion. Since there was not much research available on the use of external spin filters, it was difficult to predict the results of these experiments. Due to the greater amount of control over the forces felt by the cells, however, it was believed that the external spin filter

system would lead to the greatest maximum cell concentrations and the greatest productivities of monoclonal antibodies.

2.3 Experimental Design – Cell Lines and Medium

Two suspended mammalian cell lines that produced monoclonal antibodies were studied in this thesis project: a Chinese hamster ovary cell line referred to as "HPCHO" and a hybridoma cell line referred to as "IB4." These cell lines and the medium necessary to grow these cell lines are detailed below.

The HPCHO cell line was a Chinese hamster ovary cell line producing monoclonal antibody immunoglobulin G subclass 1 (IgG_1). A 50-50 mixture of BD CHO Medium (BD Biosciences, MD) and Sigma-Aldrich Ex-Cell Animal Component Free CHO Medium (Sigma-Aldrich Corp., MO) was used in all experiments for this cell line. Additives to the BD CHO medium included penicillin-streptomycin (100 IU/mL and 0.1 mg/mL, respectively, Sigma-Aldrich Corp., MO), L-glutamine (2mM), sodium pyruvate (1mM), and Pluronic-F68 (0.1% w/v, Sigma-Aldrich Corp., MO). Additives to the Sigma-Aldrich Ex-Cell medium included penicillin-streptomycin (100 IU/mL and 0.1 mg/mL, respectively, Sigma-Aldrich Corp., MO), L-glutamine (4mM), and sodium pyruvate (1mM).

The IB4 cell line was a mouse-mouse hybridoma cell line producing monoclonal antibody immunoglobulin G subclass 2a (IgG_{2a}). BD Cell mAb Medium Quantum Yield (BD Biosciences, MD) was used in all experiments for this cell line. Additives included 10% w/v Fetal Bovine Serum (Hyclone, UT), penicillin-streptomycin (100 IU/mL and 0.1 mg/mL, respectively, Sigma-Aldrich Corp., MO), L-glutamine (2mM), sodium pyruvate (1mM), and Pluronic-F68 (0.1% w/v, Sigma-Aldrich Corp., MO).

To prepare for inoculation of all experimental systems , 1 mL samples of the cell lines from cryovial that had been frozen in liquid nitrogen were thawed and resuspended with fresh medium into 75 cm^2 tissue culture flasks and stored inside an incubator (5% CO_2, 37°C, 90% humidity). Once the cells fully recovered from liquid nitrogen storage (the cell viability maintained a level above 90%), the cell lines were grown in spinner flasks inside the same incubator and were passaged every 3 days. The cell lines were

repeatedly passaged for at least 2 weeks before experiments in bioreactors were begun. Prior to inoculation, cells in 250-mL spinner flasks were harvested by centrifugation and resuspended with fresh medium. The desired initial cell concentration of all experimental systems was 1.5 – 2.5 x 10^5 cells/mL after inoculation.

2.4 Experimental Design – Analytical Equipment

Cell density and viability were measured by an automated trypan blue cell density examination system (Cedex, Innovatis GmbH, Germany). For this reason, the term 'cell density' is used throughout this report to refer to 'cell concentration.' Growth rates were calculated from the data gathered by this examination system. The growth rate was calculated using a form of the exponential population growth equation,

$$N_f = N_0 e^{k\Delta t},$$

in which N_f was the number of viable cells in the culture after time t; N_0 was the number of viable cells at the initial time; k was the growth rate; and Δt was the time elapsed ($t_f - t_0$). Rearranging this equation to solve for k and substituting N_v and N_{tot} with viable cell density, ρ_v, and total cell density, ρ_{tot}, because the ratio between these two parameters is the same as between N_v and N_{tot}, the growth rate k was solved by:

$$k = \frac{\ln \rho_f - \ln \rho_0}{\Delta t}$$

This formula was used to solve for point growth rates, or the growth rate over the interval of time between each sampling of the reactor. The growth rate was only calculated for points that fell in the exponential growth phase of the cell culture. The average growth rate for the entire exponential growth phase was then determined by averaging the point growth rates.

Glutamine, glutamate, glucose, lactate, ammonium, sodium, and potassium concentrations were measured off-line by a NOVA BioProfile 400 (Nova Biomedical Corporation, MA), which utilized a series of membranes and electrodes. The osmolality of the culture was calculated, also by the NOVA BioProfile 400. When certain membranes or electrodes were unable to be calibrated, the corresponding metabolite

concentration was not considered in the analysis of the data. When this calibration error also effected the osmolality concentration, the osmolality value for that sample was also discarded. An external pH meter (Corning Inc., NY) was used to verify the pH of the culture off-line.

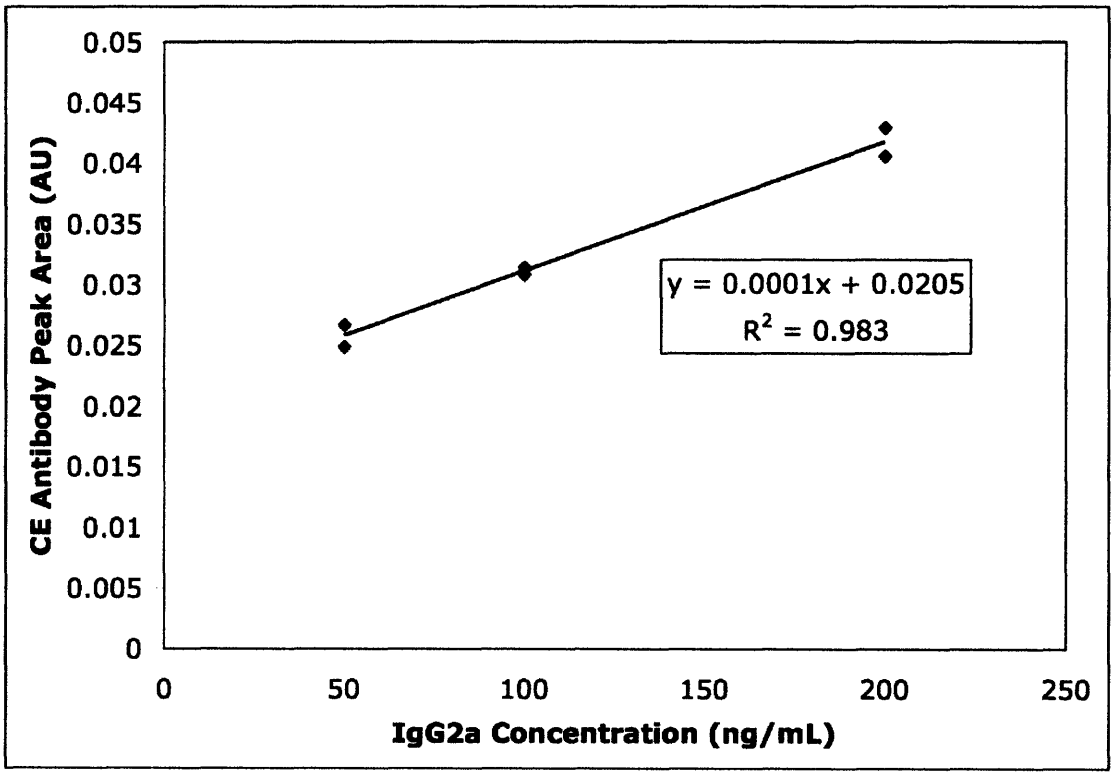

Figure 4 – Standard curve for concentration of IgG_{2a} measured by capillary electrophoresis.

The antibody content of the IB4 hybridoma cell culture was analyzed by capillary electrophoresis (Groton Biosystems, MA). The antibody content of the HPCHO cell line was not detected by this instrument and was therefore qualitatively compared by a number of variables to be discussed below. Culture samples were centrifuged at 1000 rpm for 1 minute and the supernatant was filtered through a 0.2 μm syringe filter. A filtered sample of 150 μL was mixed with 150 μL of sample buffer (CGE Protein Sample Buffer, Groton Biosystems, MA). Each sample was run on the machine with the following protocol: run buffer was delivered through the capillary for 3 minutes at 1000 mBar and no voltage, then the sample was delivered through the capillary for 0.2 minutes at 200 mBar and no voltage, and finally the capillary was placed in run buffer and a voltage of -23.3 kV was maintained for 50 minutes. The temperature of the sample and capillary electrophoresis machine was maintained at 26°C, the wavelength of the lamp

used was 214 nm, the range was 0.2 absorbance units (AU), and the time constant was 0.1 second.

A standard curve was first generated using IgG_{2a} mouse isotype control (Sigma-Aldrich Corp., MO). This standard curve is shown in Figure 4. A linear regression trend line was fit to the data and is shown in Figure 4. The R-value for this trend line was 0.983, which is a good fit. The line was not fit through the origin of the standard curve, because the capillary electrophoresis system did not detect concentrations below 10 ng/mL and the data was nonlinear below 25 ng/mL. The peak area calculated from the absorbance plot was used to estimate the concentration of the antibody. An example of the most concentrated antibody sample from the perfusion experiment involving the stirred ceramic membrane reactor system is shown in Figure 5.

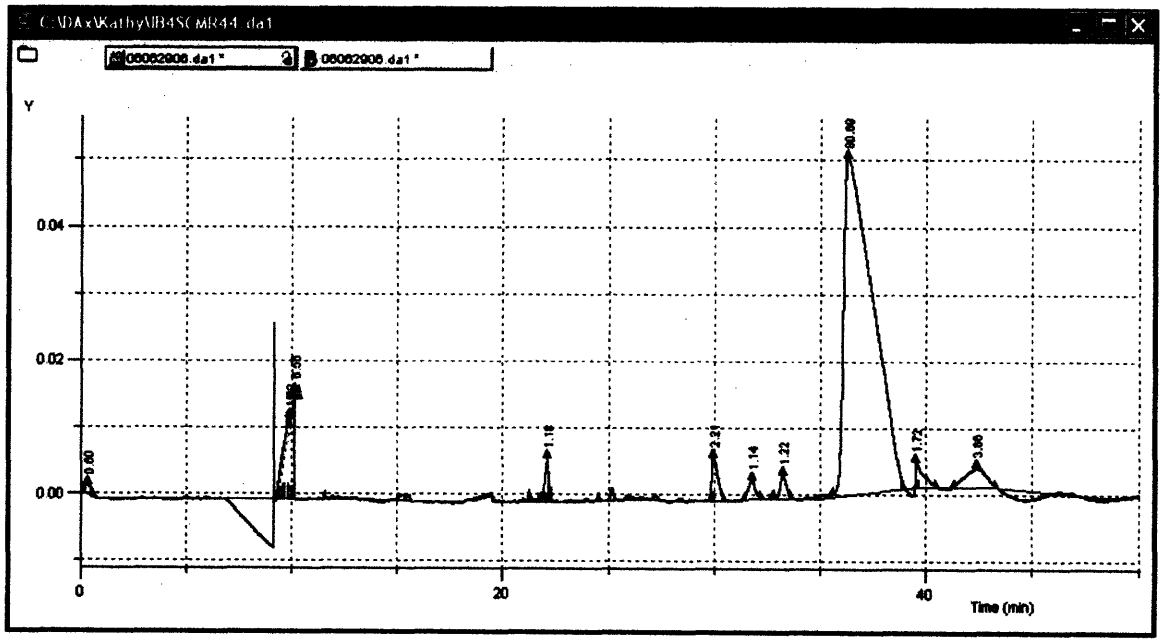

Figure 5 – Example of absorbance data generated by capillary electrophoresis analysis of an IgG_{2a} sample from the perfusion experiment involving the stirred ceramic membrane reactor.

An attempt was made to evaluate the reproducibility of the capillary electrophoresis data. A sample of 100 ng/mL was analyzed and the area of a sample peak determined seven times. The average value was 0.0009397 AU and the standard deviation was determined to be 0.0001417 AU. The standard deviation of the seven samples was found to be about 15% of the average value of the seven samples. Unfortunately, further quantification of the reproducibility and accuracy of the capillary

electrophoresis instrument was not possible due to blockage of the capillary used for experimental analysis. Changing this capillary would have altered the conditions under which the experimental data was analyzed, and would have produced reproducibility and accuracy data that would not have been reflective of the analysis of the experimental samples. Since further quantification was not possible, error bars representing 15% of the experimental values will appear in the capillary electrophoresis data below.

2.5 Experimental Design – Feeding Strategies

A batch run was performed for each cell line as a base case for comparing the fed-batch and perfusion systems. The bioreactor used was a 3-L (1.8-L working volume) Applikon glass autoclavable bioreactor (Applikon Biotechnology B.V., Netherlands) with two three-fin pitched-blade impellers. An ADI 1030 Bio Controller (Applikon Biotechnology B.V., Netherlands) was used to control temperature, dissolved oxygen, and pH using proportional integral derivative (PID) control of all three culture conditions. During batch culture, the air flow rate to the reactor was set at 0.1 vvm. The dissolved oxygen (DO) value was maintained at 50% of air saturation by sparging of O_2. The pH of the culture was maintained at 7.0 with the combined addition of an aqueous solution of 1M NaOH and the sparging of CO_2. The agitation of the culture was kept at 100 rpm. The temperature of the culture was maintained at 37°C using a heat jacket. The inoculation density of each batch run was $1.5 - 2.5 \times 10^5$ cells/mL and the working volume was 1.8 L. Sampling with the analytical equipment described in the previous section was performed twice daily, once in the morning and once in the evening. The sampling line was purged with 3 mL of culture and then 5 mL of culture was taken from the reactor to be analyzed.

Fed-batch experiments were performed for each cell line. The goal of these experiments was to develop an optimized feeding strategy to increase the maximum density and the monoclonal antibody productivity of the cells over the standard batch case. Time and equipment limitations were strong factors in the number of fed-batch experiments that were ultimately completed. In each fed-batch experiment, the same bioreactor, controller, and conditions were used as in the batch experiment, except that

the initial working volume was 1.6 L for the HPCHO cells and 1.65 L for the IB4 hybridoma cells. Concentrated basal medium was used for feeding the culture and was prepared using a Rota-Vapor apparatus (Büchi Labortechnik AG, Switzerland). For the HPCHO cells, 1 L of BD CHO Medium (BD Biosciences, MD) was concentrated to 250 mL. For the IB4 hybridoma cells, 1 L of BD Cell mAb Medium Quantum Yield (BD Biosciences, MD) was concentrated to 300 mL. The first feeding for both cell lines was initiated after about 3 days, when the glutamine concentration was reduced to about a third of its original value, the glucose concentration was reduced to about half of its original value, and the concentrations of lactate and ammonium ion had rapidly increased. Various later time points for feeding and additives to the concentrated feed mediums were considered.

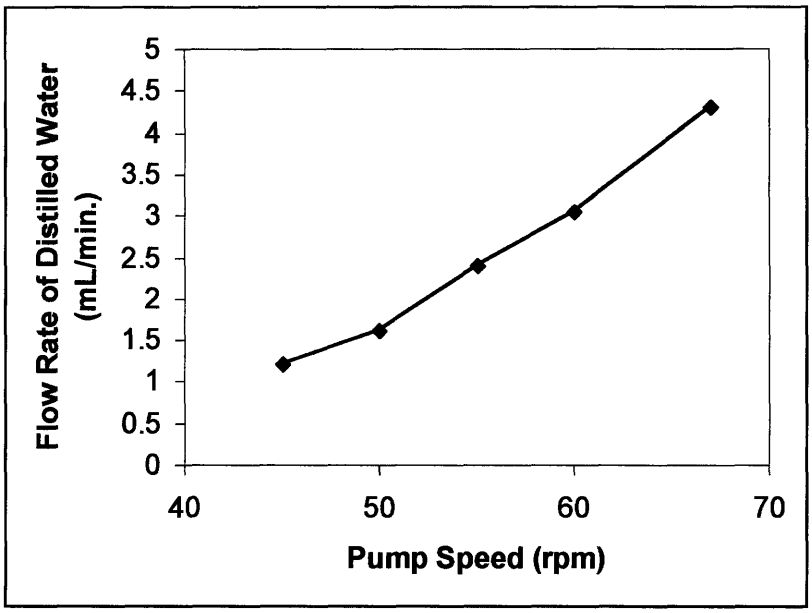

Figure 6 – Flow rates of distilled water for various pump speeds on dual-headed Masterflex peristaltic pump.

Perfusion experiments were performed for each cell line, using three different cell retention devices that will be described in the next section. The goal of these experiments was to find the optimal cell retention device and feeding strategy to increase the maximum density and productivity of the cells over the standard batch and experimental fed-batch modes. Again, time and equipment limitations were strong factors in the number of perfusion experiments that were ultimately completed. In each perfusion experiment, the same bioreactor, controller, and initial conditions were used as in the

batch experiment. The reactor was inoculated in the same manner as the batch runs, but after about 70 hours, the cell retention device was initiated and the perfusion feeding strategy begun. At the 70 hour point, the cell culture was at about 2/3 of the maximum density of the batch experiment and there were significant decreases in glucose and glutamine concentrations and significant increases in lactic acid and ammonium concentrations. The medium used in the perfusion feeding was the same basal medium with the same supplementations as described above for the batch experiments. Feeding and harvesting was controlled by a dual-headed peristaltic pump (Masterflex, Cole-Parmer Instrument Company, IL) that was calibrated with distilled water. The flow rates for various pump speeds are illustrated in Figure 6. Autoclaved 3 liter Nalgene bottles (Thermo Fisher Scientific Inc., MA) were used to supply the fresh medium and collect the spent medium. The feeding and harvesting rate began at about 0.5 working volumes / day and then increased daily by 0.5 working volumes / day to a final rate of about 3 working volumes / day. If there were significant losses of glucose and glutamine concentrations to the culture or significant increases in lactic acid or ammonium concentrations to the culture, the feeding and harvesting rates were increased twice daily instead of once daily. The culture was continued until the cell retention device was fouled, ending flow to the harvest bottle, or when the viability of the cell culture decreased below 75%.

2.6 Experimental Design – Cell Retention Devices

Three different cell retention devices were utilized in the perfusion experiments. One of these devices, the stirred ceramic membrane reactor (SCMR), was an internal cell retention device. The two other devices, the alternating tangential flow hollow fiber membrane (ATF) and the external spin filter (ESF), were external cell retention devices. The three methods of cell retention are described in more detail and accompanied by schematics below.

One method of perfusion culture involves inserting a system of ceramic membranes into a stirred tank reactor. This stirred ceramic membrane reactor (SCMR) system has been used to obtain a dense cell culture of *Saccharomyces cerevisiae* with a

cell mass concentration or 207 g/L (25). A similar SCMR system utilizing asymmetric porous ceramic tubes was used to maintain a high filtrate flux and high cell density (141 g/L with 94% viability) of *Lactococcus lactis* (26). Yield and productivity were improved by optimizing the feeding of fresh medium and filtering the supernatant of the dense cell culture. The SCMR system has been used for continuous production of lactic acid from molasses by dense perfusion culture of *Lactococcus lactis* (23). In addition, the SCMR system utilized in this thesis has been used to study the effects of increasing rates of perfusion rate on the cell growth of IB4 suspension hybridoma cells and the IgG_{2a} productivity of the cells (24). An optimal feeding strategy was found and led to a maximum density of 350 x 10^5 cells/mL, a high mAb volumetric productivity (690 mg/L-d), and a high yield (340 mg/L). This paper, however, did not evaluate the effect of the presence of the ceramic membranes inside the reactor on the health and productivity of the cells, used serum-free medium, and did not involve comparisons to other types of perfusion systems.

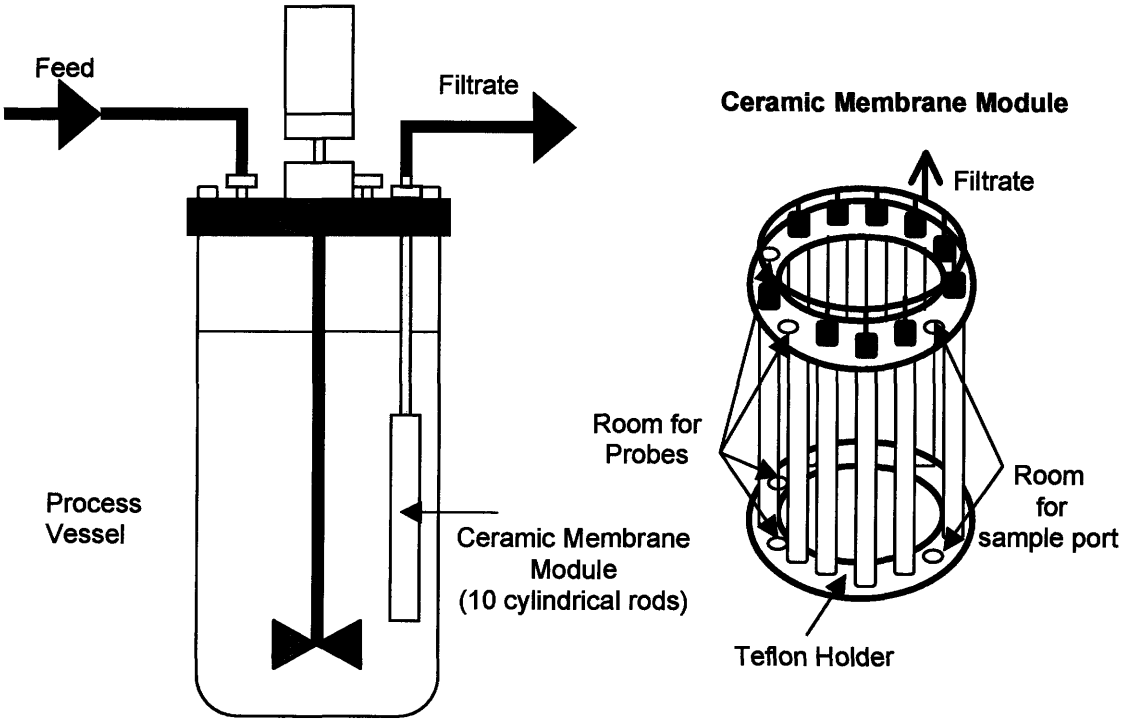

Figure 7 – The stirred ceramic membrane reactor system, consisting of a ceramic membrane module of 10 cylindrical rods inserted into a typical bioreactor set-up (adapted from figure by Alyse Wu).

A schematic of the SCMR system is shown in Figure 7. This system was comprised of ten ceramic cylinders, connected by tubing and inserted into the bioreactor.

The ceramic material withstood high temperature and pressure well, so the bioreactor was autoclaved with the ceramic membrane module inside the bioreactor with a working volume of phosphate buffer saline solution. Each porous ceramic cylinder (Cefilt; NGK Co. Ltd., Japan) was made of Al_2O_3 with a mean pore size and thickness of 25 μm and 1.3 mm at the inner layer and 0.2 μm and 0.2 mm at the surface layer, respectively. Each ceramic cylinder had an inner diameter of 8 mm, and outer diameter of 11mm, and a length of 115 mm. The total active surface area of the filter module, or the ten ceramic cylinders connected together, was approximately 400 cm^2. The filter unit was connected to tubing that led to the head plate of the reactor and then to the harvest line, which was controlled by a peristaltic pump. A second pump head on the same peristaltic pump was used to feed medium drop-wise into the reactor from a feeding line. The ceramic filter provided a 4.0 mL/h-cm^2 flux for drawing distilled water. Experiments were carried out as described in the previous section. A control experiment in which the filter module was placed in the reactor for a batch run (with no feeding or harvesting of medium) was performed for each cell line.

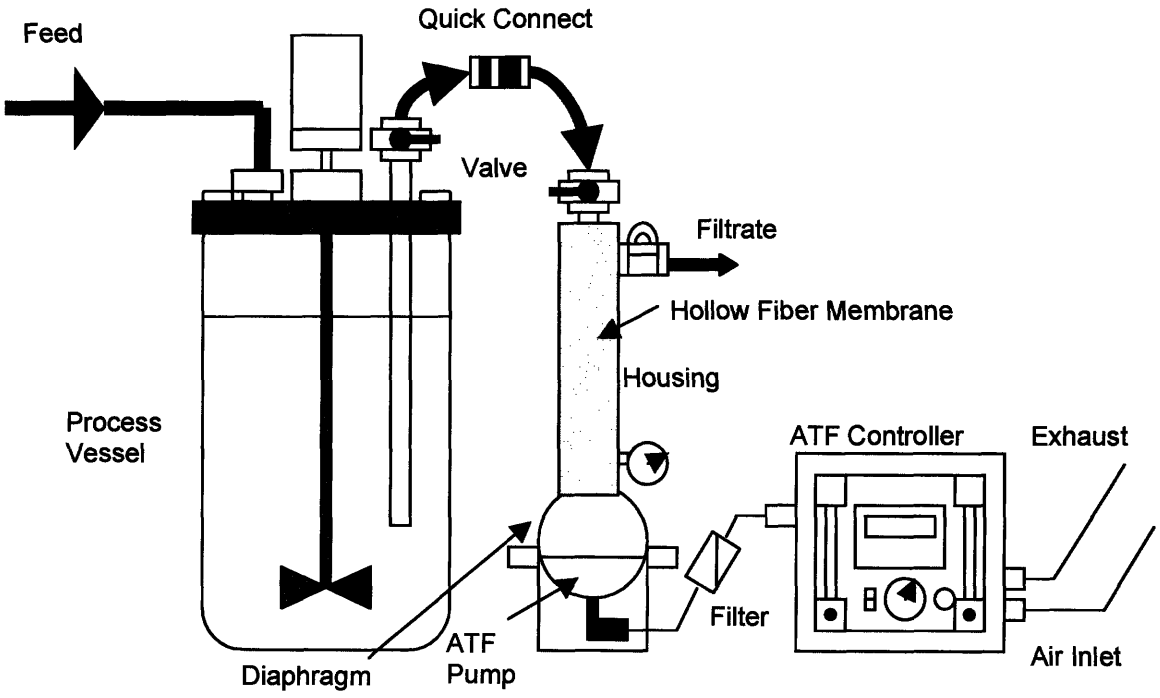

Figure 8 – The ATF system, consisting of the controller, pump, housing, filtration device, joint assembly (connects the housing to the vessel), and typical bioreactor set-up. Pressurization and exhaust cycles alternate according to specified time sequences (adapted from figure by Alyse Wu).

Another method of perfusion culture is called the alternating tangential flow (ATF) filter system (Refine Technology, Co., NJ) (12, 13). A schematic of this system is shown in Figure 8. The ATF has three principal components: a hollow fiber filter cartridge (0.2 μm pore size), the ATF diaphragm pump, and the ATF controller. The pump generates alternating tangential flow through the hollow fibers, reversing the fluid flow through the filter by changing pressure differences across the hollow fiber cartridge. This is accomplished by dividing the pump into two chambers with a flexible, medical-grade silicone diaphragm. The ATF controller cycles filtered air to and from one of the pump chambers, generating a positive of negative pressure gradient relative to the bioreactor and reversing the fluid flow through the hollow fiber membrane. This alternating tangential flow reduces the likelihood of filter fouling, a major disadvantage of using filter systems for perfusion culture. The cell-free filtrate is harvested by a peristaltic pump while the cells are returned to the reactor. Fresh medium is added to the reactor using another pump head on the same peristaltic pump but a separate line. Sterilization is possible because the hollow fiber filter cartridge is autoclave safe. The ATF system has been used to grow mammalian cells to densities of over 10^7 cells/mL (12). The ATF system has also been used in fed-batch investigations to concentrate culture before fresh medium was added.

In this thesis, the ATF system was used with both CHO cells and hybridoma cells in an effort to produce higher yields of monoclonal antibodies than was possible with other systems. The set-up of the system was as described above. It was necessary to determine pressure conditions and timing that best retained cells of each cell line without significantly damaging the cells in the process. The ideal pressure set point of the ATF controller was determined to be about 4 psi and the exhaust cycle lasted approximately 10 seconds. Experiments were carried out as described in the previous section.

A final method of perfusion culture involves the use of a spin filter. There are two types of spin filters: internal and external. Internal spin filters consist of a cylindrical metal wire mesh that spins on the impeller shaft of the bioreactor. The spinning of the mesh generates a centripetal force that forces cells to the outside walls of the reactor and allows smaller particles in the media, such as metabolites, to pass through the wire mesh. The particles that pass through the mesh are harvested from the reactor while the cells are

retained inside the reactor and fresh medium is added to the reactor through a different line. In an external spin filter system, the same principles apply, but the spin filter is not contained inside the bioreactor. Cell suspension is harvested from the bioreactor by a peristaltic pump and fed into the base of a glass vessel containing the external spin filter. The cells are kept against the vessel wall by centripetal force and are returned to the bioreactor, along with fresh medium. The smaller particles in the media are harvested from a line inside the wire mesh. Since the spin filter is not mounted on the impeller of the bioreactor, different speeds may be used to agitate the cells in the bioreactor and to separate them from media components in the spin filter.

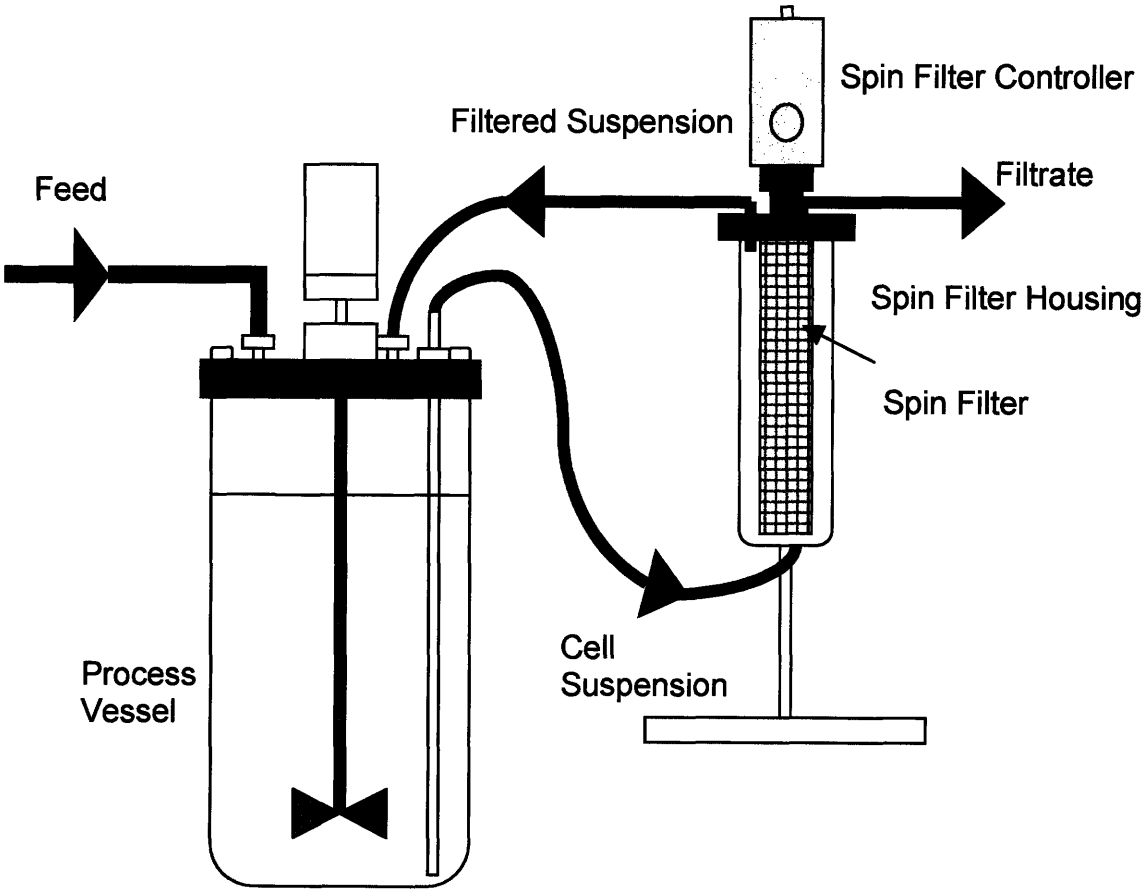

Figure 9 – The ESF 100 G external spin filter system, consisting of a filter element, stand, rotor, and typical bioreactor set-up (adapted from figure by Alyse Wu).

There are many papers in the literature concerning the use of internal spin filters (16, 27), but external spin filters represent a relatively new method that has not been researched extensively. It has been found that scaling up the size of a spin filter does not affect the fluid exchange flow per unit bioreactor volume because the screen surface area

per bioreactor volume decreases (27). Since the fluid exchange flow per unit bioreactor volume is not increased with scale-up, it can be assumed that the risk of filter fouling will not increase with scale-up and that cell retention will not decrease with scale-up. It has been shown that at high spin speeds, fouling of the filter is prevented but the efficiency of cell retention is decreased. It has been shown that at low spin speeds, however, the efficiency of cell retention is increased while the incidence of filter fouling is increased (27). It is therefore necessary to find an optimal spin speed that maximizes cell retention but minimizes filter fouling. The use of external spin filters may make this goal possible because the speed of the external spin filter module is not limited by the rate of agitation in the bioreactor.

In this project, the ESF 100 G (Sartorius BBI Systems, Germany) was used. A picture of this system is shown in Figure 9. The system consists of a rotating spin filter element mounted on a stand and controlled by a rotor. The spin filter element is a closed rotating sleeve carrying a multilayer stainless steel mesh of 20 μm. The rotor can spin the element from 1 rpm to 2000 rpm, although the stand is only stable up to about 500 rpm. At 350 rpm, the flow in the filter element becomes turbulent, thus improving cell retention. It was necessary to determine the optimal rotation speed of the spin filter for perfusion of each cell line. A peristaltic pump with two heads was used to feed fresh medium to the reactor and extract medium from the external spin filter apparatus. An additional peristaltic pump was used to extract cell culture from the reactor at 200 rpm and deliver the material to the base of the external spin filter apparatus. Sterilization was possible because the entire spin filter apparatus may be autoclaved and aseptically connected to the bioreactor. Experiments were carried out as described in the previous section.

3 Experiments with HPCHO Chinese Hamster Ovary Cells

3.1 Batch system

A batch experiment was performed with the HPCHO Chinese hamster ovary cell line in the manner described above. The three-liter bioreactor was inoculated with a 1.8-liter working volume of cell culture at a density of around 2.5 x 10^5 cells/mL. The only disturbance to the culture was the purging of the sample line of 3 mL and the sampling of 5 mL twice daily. The total amount of medium supplied to the HPCHO batch experiment was 1.8 liters.

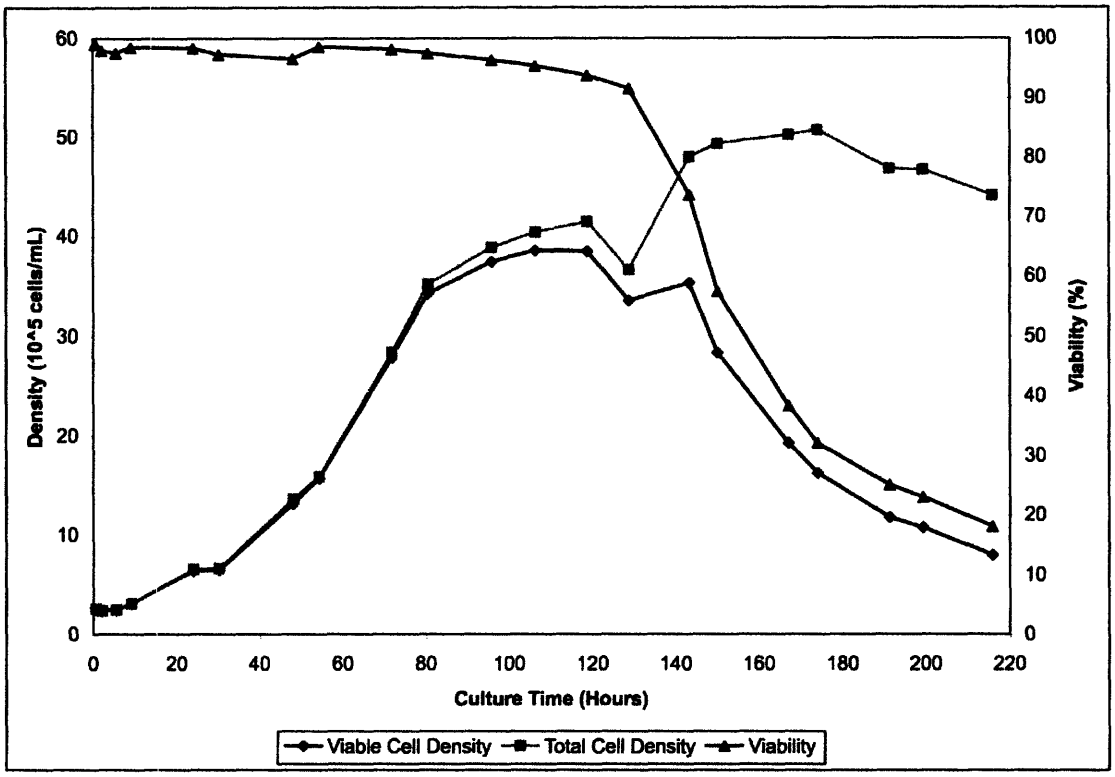

Figure 10 – Growth Data for HPCHO Batch Run

The cell concentration and viability data for the batch experiment are shown in Figure 10. The culture experienced a lag phase until around 30 hours. During this time, the CHO cells were adjusting to the change in environment resulting from transitioning from the spinner flask to the stirred tank reactor. Exponential growth of the culture began around 30 hours and continued until around 80 hours. The average growth rate during

this period was 0.026/hr. The maximum growth rate during this period was 0.058/hr and occurred at the end of the exponential growth phase around 75 hours. The culture experienced a stationary phase from around 80 hours to about 120 hours. A relatively long death phase began around 120 hours and continued until about 215 hours, when the viability of the cells dropped below 20% and the experiment was ended.

The maximum viable cell concentration reached by the batch CHO culture was 38.62 x 10^5 cells/mL at 105 hours. The maximum total cell concentration achieved was 50.72 x 10^5 cells/mL at 175 hours. The viability of the culture was very high throughout the lag, exponential growth, and stationary phases. The viability of the cells remained above 95% until 105 hours and remained above 90% until 130 hours. The cells began dying around 120 hours. For fed-batch and perfusion culture of these CHO cells, it was determined that feeding of additional nutrients should begin prior to 120 hours.

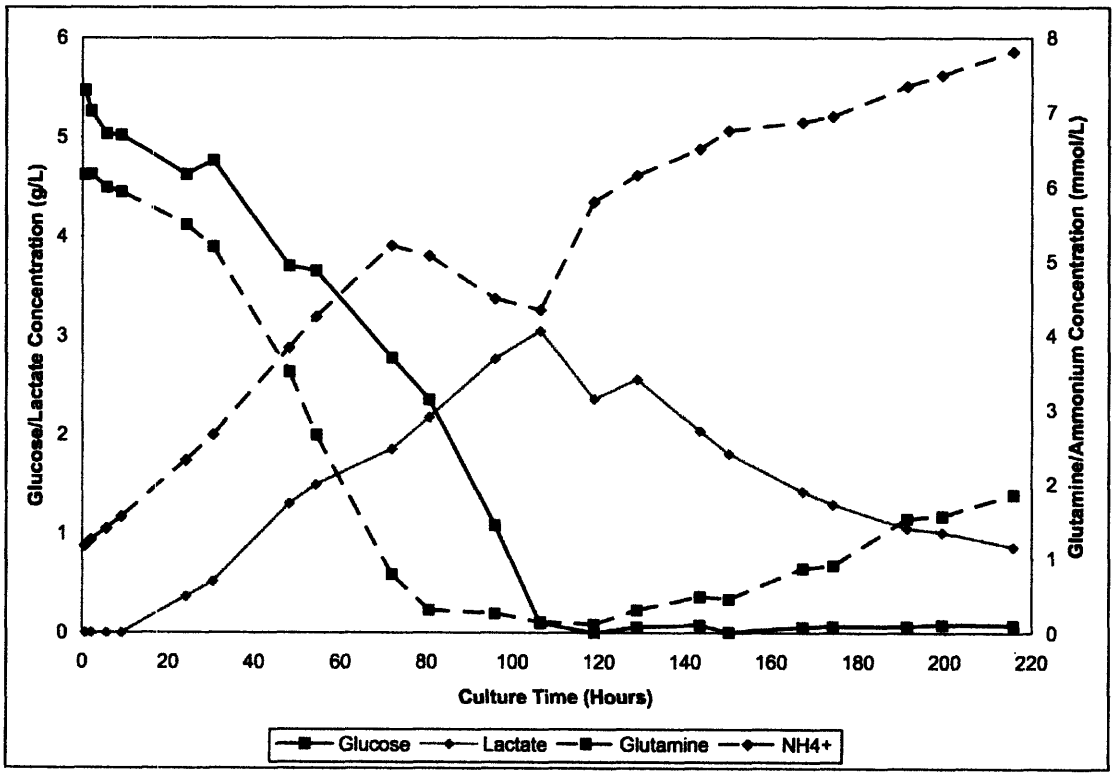

Figure 11 – Metabolic Data for HPCHO Batch Run

The major metabolite concentrations of the batch CHO culture are shown in Figure 11. The glucose concentration began at around 5.5 g/L, and was reduced to 0 g/L by 120 hours. The lactate concentration began at 0 g/L and increased to over 3 g/L by 105 hours. After this point, the lactate concentration decreased until it was around 1 g/L

at 220 hours. The increases in lactate concentration corresponded to the decreases in glucose concentration. The lactate concentration stopped increasing as soon as the glucose concentration fell to significantly low levels. The glutamine concentration began at around 6 mmol/L and was reduced to below 0.2 mmol/L by 105 hours. The concentration of ammonium ion began at around 1 mmol/L and increased until it reached nearly 8 mmol/L at 220 hours. There was a slight decrease in ammonium ion concentration from 80 to 105 hours, possibly due to the fact that the glutamine concentration fell to significantly low levels at this point. The ammonium ion concentration begins to increase again after 105 hours, most likely due to death of the cells. It appears that the culture utilized glutamine at a faster rate than it used glucose and produced ammonium ion at a faster rate than it created lactate. This observation is incorrect, however, because less glutamine than glucose was initially available to the culture. The two metabolites may be used for two purposes. Glutamine may serve to jump start the growth of the cells and glucose may be more useful for division and the productivity of cells. From the metabolite concentration data, it is apparent that for the fed-batch and perfusion experiments, feeding should begin prior to 80 hours, or the point at which glutamine concentrations have decreased significantly.

Because the capillary electrophoresis assay was not reliable for analyzing the productivity of the HPCHO cell line, it is only possible to predict the productivity of the cells and make qualitative comparisons between the batch experiment and the experiments involving other feeding strategies. It has been found that the specific antibody production rate of the H22 hybridoma cell line in perfusion culture is strongly related to the viable cell density of the culture (28). This correlation has been found, even though the substrate consumption and product formation rates of metabolites were strongly related to specific growth rate (28). Although the HPCHO cells were not a hybridoma cell line like H22, they produced a similar product and it was assumed that the specific antibody production of the HPCHO cells correlated to the viable cell density of the culture. It could be argued that the viable cell concentration data for the batch experiment, therefore, establishes a base case scenario of antibody production for a comparison of the production achieved by the various feeding strategies.

From discussions with D.I.C. Wang about productivity of CHO cells (29), researchers have noticed that CHO cells produce more antibodies when the cells are placed under stress. This information is substantiated by a study involving humanized antibody production in CHO cells (30). When a single stressful culture condition is applied to the cell culture system (osmotic stress or low temperature, for example), the specific production of erythropoietin by a recombinant CHO cell line was enhanced 1.8- to 2.2-fold. When a rapid increase in the osmolality was induced in a culture of CHO cells, there was inhibited cell growth and increased mAb production (31). There is also evidence that an increase in osmolality in cell culture medium can lead to an increase in diameter of the cells (32). It is a logical assumption, then, that cells in an environment with a high osmolality that stop doubling but continue to grow larger are probably taking up nutrients for the purpose of antibody production rather than division (29).

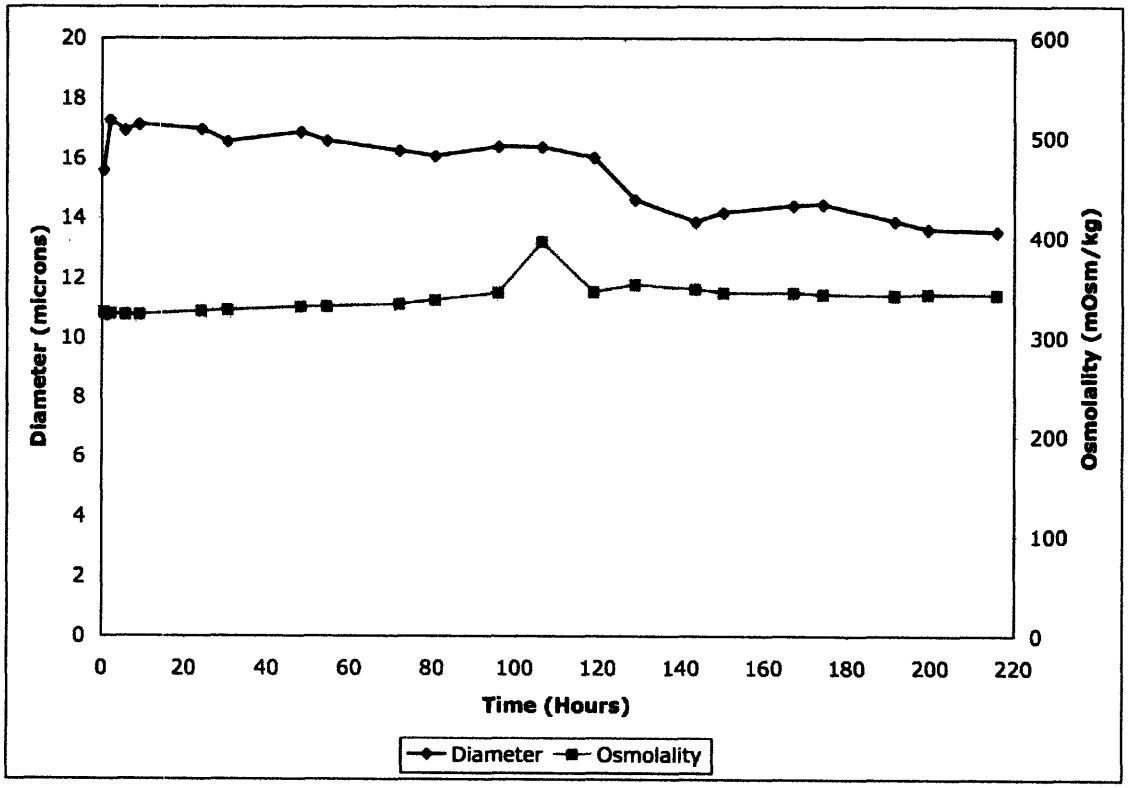

Figure 12 – Diameter and Osmolality Data for the HPCHO Batch Run

Because of this assumption, the diameter of the HPCHO cells and the osmolality of the medium in the batch experiment were investigated. These data appear in Figure 12. Initially, the diameter of the HPCHO cells in the batch experiment was relatively

constant and was in the 16 to 17 micron range. When the viability of the culture began to drop below 90% after about 120 hours, the diameter of the cells decreased to the 13.5 to 14.5 micron range. There were no significant increases in cell diameter during the batch experiment. The osmolality of the cell culture medium was over 320 mOsm/kg, which is a bit high for CHO medium (29). The osmolality increased slightly until 105 hours, when there was a sharp peak of 400 mOsm/kg. This may correspond to a peak in production by the cells, according to the assumptions described above. The osmolality of the medium then decreased and leveled out around 340 mOsm/kg. With the viable cell concentration data, the cell diameter and osmolality data was used to qualitatively compare the production of the HPCHO cells in the batch mode to that of other feeding strategies.

3.2 Fed-batch system

Several fed-batch experiments were performed with the HPCHO Chinese hamster ovary cell line as described above. Initial fed-batch experiments were performed with various starting volumes ranging from 1 L to 1.65 L of a 50-50 mixture of BD CHO Medium (BD Biosciences, MD) and Sigma-Aldrich Ex-Cell Animal Component Free CHO Medium (Sigma-Aldrich Corp., MO). Each medium was supplemented as previously described. It was found that 1.65 L of medium allowed the cells to grow in a matter most similar to that in the early stages of the batch experiment. This was due to the need for the volume of the culture to be great enough to completely immerse the two sets of impeller blades on the drive shaft of the reactor. These initial experiments also showed that feeding of the culture should proceed after about 72 hours of growth and could be supplied in around 100 mL bulk doses via a sterile syringe.

The next fed-batch experiments involved varying the composition of the feed medium. A 50-50 mixture of supplemented BD CHO medium concentrated via Rota-Vapor apparatus from 1 L to 250 mL and supplemented Sigma-Aldrich Ex-Cell Animal Component Free CHO medium concentrated via Rota-Vapor apparatus from 1 L to 350 mL was first utilized. As soon as this medium was added to the culture, glucose and glutamine uptake by the cells halted, the ammonium ion concentration increased

significantly, and the cells quickly began dying. Next, supplemented BD CHO medium concentrated via Rota-Vapor apparatus from 1 L to 250 mL was used as the only feed medium. Again, glucose and glutamine uptake by the cells halted, the ammonium ion concentration increased, and the cells began dying.

The first relatively successful fed-batch experiment with the HPCHO Chinese hamster ovary cell line utilized BD CHO medium that was only supplemented with penicillin-streptomycin (100 IU/mL and 0.1 mg/mL, respectively), sodium pyruvate (1mM), and Pluronic-F68 (0.1% w/v) as the feed medium. Again, this medium was concentrated via Rota-Vapor apparatus from 1 L to 250 mL. The three-liter bioreactor was inoculated with a 1.65-liter working volume of HPCHO culture with an initial density of around 1.5 x 10^5 cells/mL. Disturbances included the purging of the sample line of 3 mL and the sampling of 5 mL twice daily, as well as two 120 mL bulk doses of feed medium delivered via sterile syringe at around 78 and 95 hours. When concentrated medium is considered, the total amount of medium supplied to the first HPCHO fed-batch experiment was 2.65 liters.

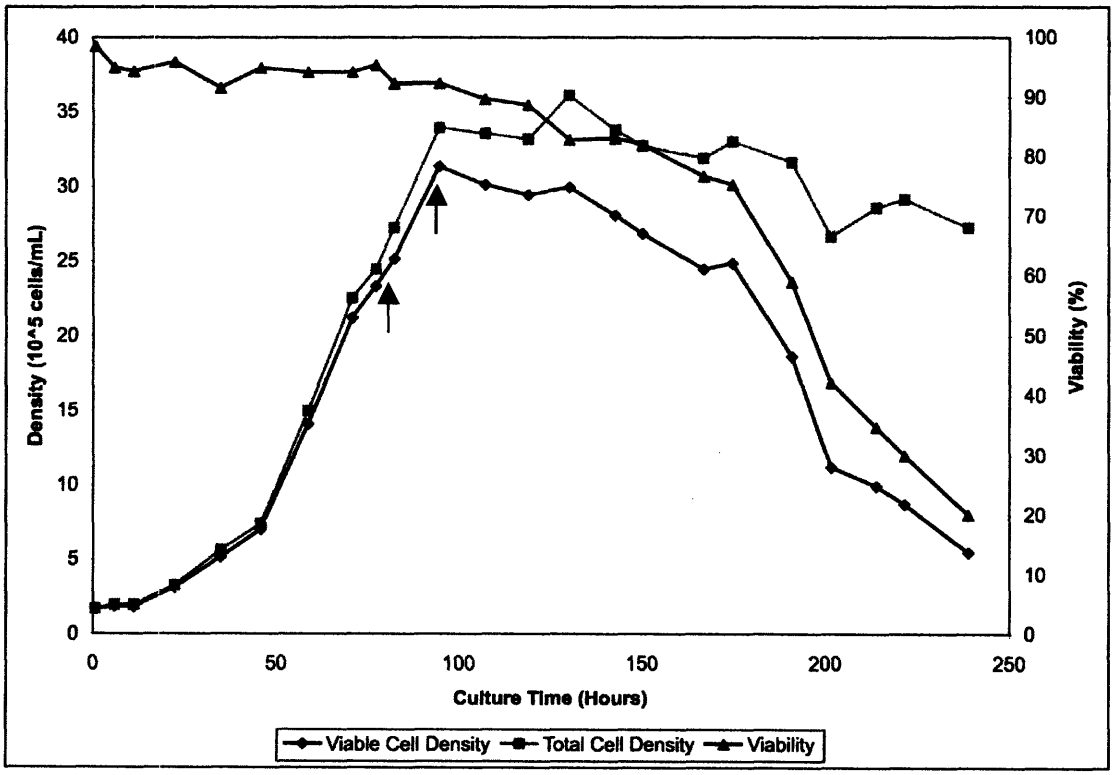

Figure 13 – Growth Data for HPCHO Fed-Batch No. 1. The black arrows indicate feeding doses.

The cell concentration and viability data for this fed-batch experiment are shown in Figure 13. The culture experienced a lag phase until around 45 hours. During this time, the CHO cells were adjusting to the change in environment resulting from transitioning from the spinner flask to the stirred tank reactor. This period was probably longer in the fed-batch experiment than in the batch experiment for two reasons. First, the initial cell concentration in the reactor was lower in the fed-batch experiment than in the batch experiment (1.5×10^5 cells/mL rather than 2.5×10^5 cells/mL). Second, the environment in the reactor was probably harsher in the fed-batch experiment due to the decreased working volume of medium. This difference in the length of the lag phase also caused the start and end of the exponential growth phase of the fed-batch and batch cultures to differ by about 15 hours. A graph showing the cell concentration data for both the batch and fed-batch CHO experiments is shown in Figure 14, so that the two experiments may be compared visually. The fed-batch data series have solid lines and the batch data series have dashed lines.

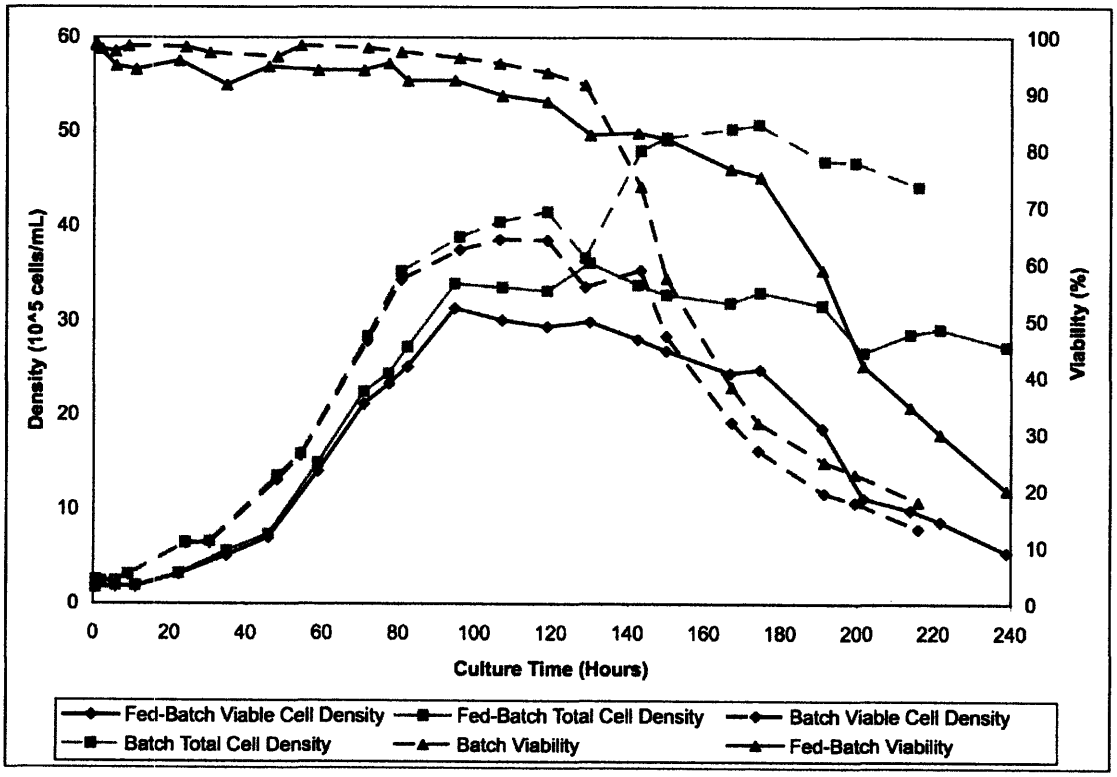

Figure 14 – Comparison of Growth Data for HPCHO Batch Run and HPCHO Fed-Batch Run No.1

Exponential growth of the fed-batch culture began around 45 hours and continued until around 95 hours. The average growth rate during this period was 0.029/hr. The

maximum growth rate during this period was 0.053/hr and occurred in the middle of the exponential growth phase around 60 hours. The average growth rate and maximum growth rate of the exponential phase of the fed-batch culture was comparable to that of the batch culture, showing that the increased lag phase was the only significant difference between the initial stages of the two experiments. The fed-batch culture experienced an extended stationary phase from around 95 hours to about 175 hours. This phase was the most interesting aspect of the fed-batch experiment and will be discussed below in the discussion of the metabolite concentrations of the culture. Finally, the fed-batch experiment experienced a death phase that began around 175 hours and continued until about 250 hours, when the viability of the cells dropped below 20% and the experiment was ended.

The maximum viable cell concentration reached by the fed-batch CHO culture was 31.37×10^5 cells/mL at 95 hours. The maximum total cell concentration achieved was 36.13×10^5 cells/mL at 130 hours. Both of these values are significantly lower than what was achieved in the batch culture (a maximum viable cell concentration of 38.62×10^5 cells/mL at 105 hours and a maximum total cell concentration of 50.72×10^5 cells/mL at 175 hours) and this fact is apparent from Figure 14. Therefore, if the productivity of this fed-batch culture were estimated purely from a viable cell density standpoint, the first fed-batch culture produced slightly less monoclonal antibody than the batch experiment. The viability of the fed-batch culture was high throughout the lag, exponential growth, and stationary phases. The viability of the cells remained above 90% until 100 hours and remained above 80% until 175 hours. As Figure 14 illustrates, the viability of the fed-batch culture was consistently lower than that of the batch culture by 5-10%. The cells in the fed-batch culture began dying around 175 hours.

The major metabolite concentrations of the fed-batch HPCHO culture are shown in Figure 15. The glucose concentration began at around 5 g/L, and was reduced to 0 g/L by 150 hours. After each feeding, there was a sharp increase in the glucose concentration. The first feeding at around 78 hours produced a larger increase, but it was quickly reduced as the cells continued to grow and divide. The second feeding at around 95 hours produced a smaller increase in glucose concentration. After this point, the cells continue to rapidly take up glucose but do not continue to divide. The HPCHO cells are

probably taking up glucose and utilizing it for the production of monoclonal antibody rather than multiplying. The lactate concentration began at 0 g/L and increased to nearly 4 g/L by 150 hours. After this point, the lactate concentration slowly decreased until it was around 3 g/L at 240 hours. The increases in lactate concentration corresponded to the decreases in glucose concentration. The lactate concentration stopped increasing as soon as the glucose concentration fell to significantly low levels. It is important to note that the amount of glucose taken up by the HPCHO cells was much greater than the amount of lactate produced by the cells. The maximum levels of the two metabolites differed by more than 1 g/L. This is further evidence that the cells continued to take up glucose and used the nutrient to produce antibody rather than to undergo normal metabolism.

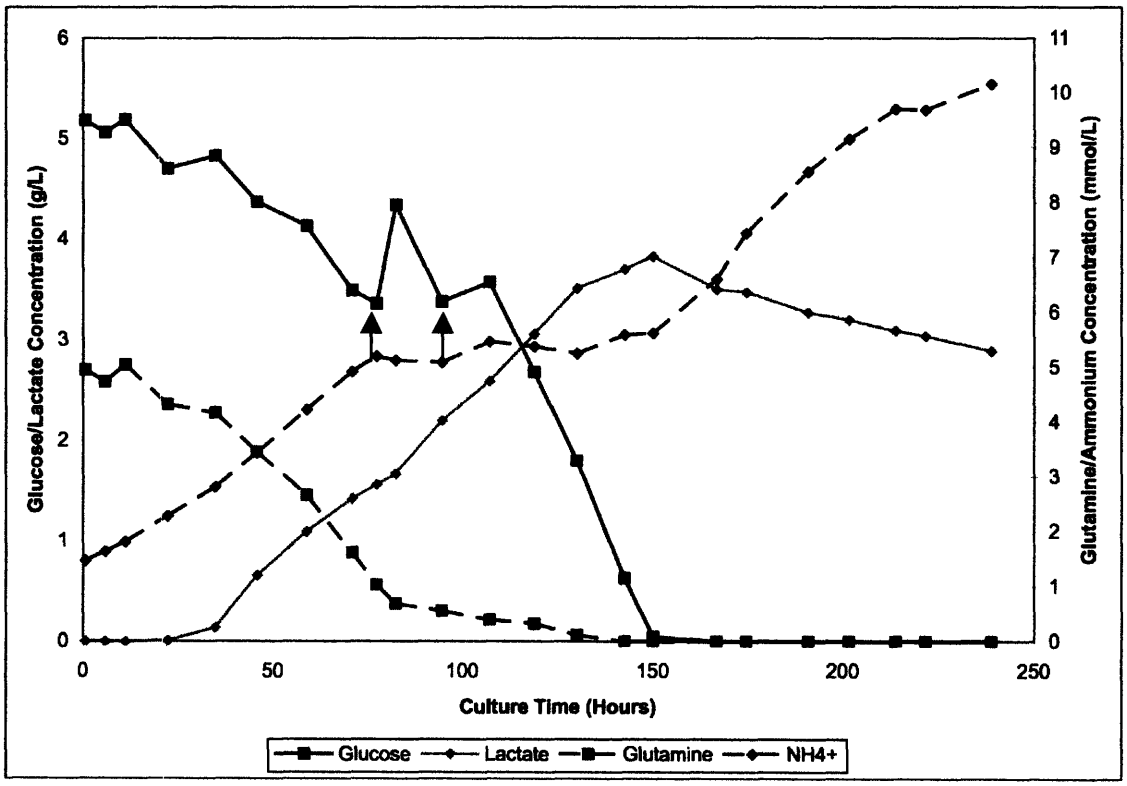

Figure 15 – Metabolic Data for HPCHO Fed-Batch Run No. 1. The black arrows indicate feeding doses.

The glutamine concentration of the first HPCHO fed-batch culture began at around 5 mmol/L and was reduced to 0 mmol/L by 140 hours. There were two regions of glutamine consumption, however. Prior to 80 hours, the glutamine concentration decreased more quickly than between 80 and 140 hours. This is further evidence that the

ultimate fate of the nutrients taken up by the HPCHO cells changed sometime between the first and second feedings of the culture. The concentration of ammonium ion began at around 1.5 mmol/L and increased until it reached over 10 mmol/L at 240 hours. The high concentration of ammonium ion in the medium could have slowed the uptake of nutrients by the CHO cells (29). The rate of increasing ammonium ion concentration decreased from 80 to 150 hours, possibly due to the fact that the glutamine concentration fell to significantly low levels at this point and was not replenished by the feed medium. The ammonium ion concentration begins to increase more rapidly again after 150 hours, most likely due to death of the cells. It appears that the culture utilized glutamine at a faster rate than it used glucose and produced ammonium ion at a faster rate than it created lactate in the beginning of the culture, when the cells were multiplying and dividing. This observation is not correct, however, because less glutamine than glucose was initially available to the culture. Once the CHO cells began using primarily glucose in an effort to mainly produce monoclonal antibody, the consumption of glutamine significantly decreased.

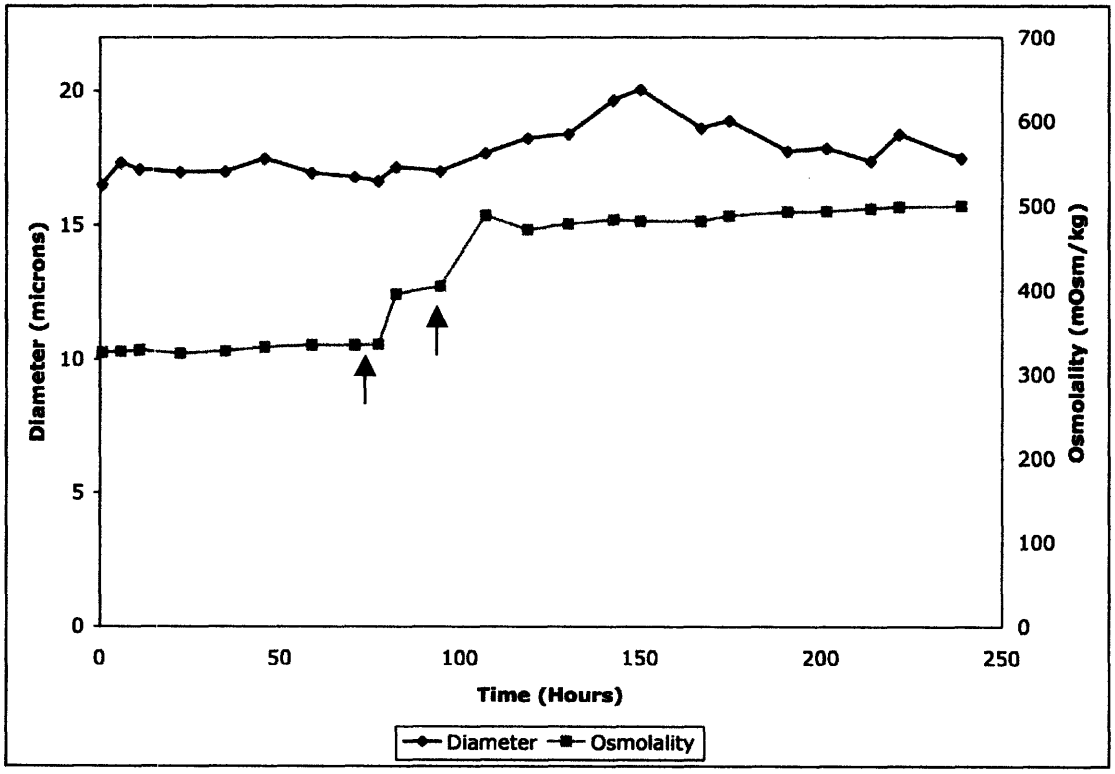

Figure 16 – Diameter and Osmolality Data for HPCHO Fed-Batch Run No.1. The black arrows indicate feeding doses

The diameter of the HPCHO cells and the osmolality of the medium in the first fed-batch culture are shown in Figure 16. The bulk doses of feed medium delivered to the first fed-batch culture of HPCHO cells produced sharp increases in the osmolality of the culture. After the first feeding at around 78 hours, the osmolality increased from about 330 mOsm/kg to about 400 mOsm/kg. After the second feeding at around 95 hours, the osmolality increased from around 400 mOsm/kg to nearly 500 mOsm/kg. The increased osmolality of the medium also corresponded to the stationary phase of the fed-batch culture, when the cells were no longer dividing, but were growing larger in diameter. It is shown quite clearly in Figure 16 that the average diameter of the HPCHO cells slowly increased from around 17 microns to over 20 microns once feeding of the culture had been initiated.

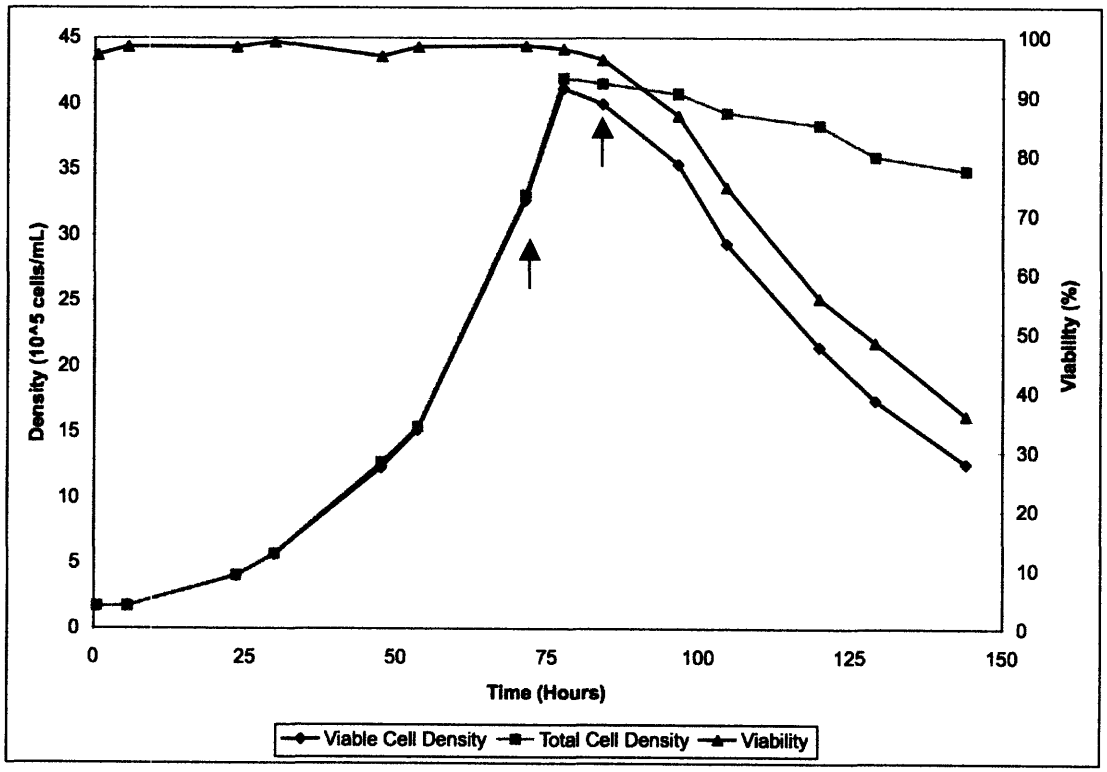

Figure 17 – Growth Data for HPCHO Fed-Batch Run No. 2. The black arrows indicate feeding doses.

As discussed in the section describing the HPCHO batch experiment, both osmolality and cell diameter may be indicative of the productivity of a culture. Therefore, the first fed-batch culture of the HPCHO cells likely produced much more than the batch culture, even though the viable cell density of the fed-batch culture was much lower than that of the batch culture. The fed-batch experiment created fewer cells,

but those cells probably produced more total monoclonal antibody than the cells in the batch experiment.

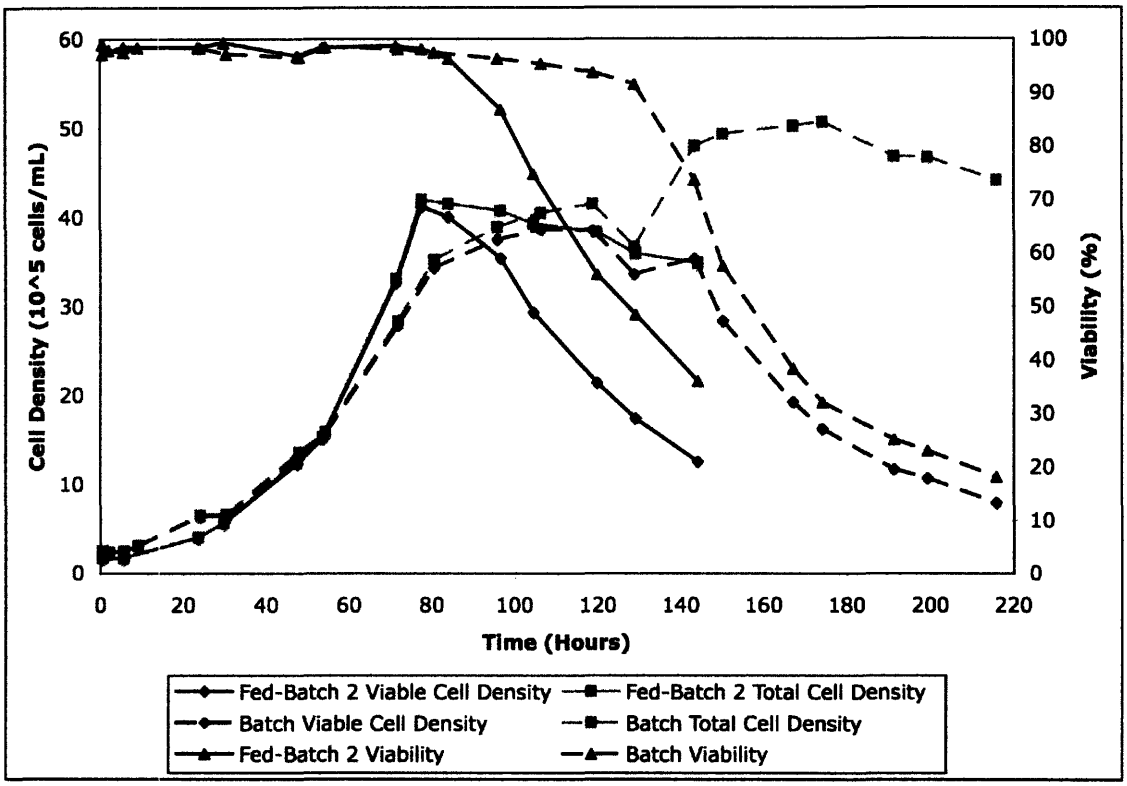

Figure 18 – Comparison of Growth Data for HPCHO Batch Run and HPCHO Fed-Batch Run No. 2

Fed-batch experiments were continued with the specific goal of increasing the maximum viable cell concentration over that achieved by the batch experiment. The second relatively successful fed-batch experiment with the HPCHO Chinese hamster ovary cell line again utilized BD CHO medium that was only supplemented with penicillin-streptomycin (100 IU/mL and 0.1 mg/mL, respectively), sodium pyruvate (1mM), and Pluronic-F68 (0.1% w/v) as the feed medium. As before, this medium was concentrated via Rota-Vapor apparatus from 1 L to 250 mL. In addition to this medium, however, concentrated essential amino acid solution (50X solution, MEM Amino Acids without L-glutamine, Sigma-Aldrich Corp., MO) was added during the feeding steps. The three-liter bioreactor was inoculated with a 1.6-liter working volume of HPCHO culture with an initial density of around 1.75×10^5 cells/mL. Disturbances included the purging of the sample line of 3 mL and the sampling of 5 mL twice daily, as well as two bulk doses of feed medium delivered via sterile syringe at around 72 and 78 hours. The first

bulk dose consisted of 110 mL concentrated supplemented BDCHO medium and 10 mL essential amino acid solution. The second bulk dose consisted of 120 mL concentrated supplemented BDCHO medium and 10 mL essential amino acid solution. When concentrated medium is considered, the total amount of medium supplied to the second HPCHO fed-batch experiment was 2.6 liters.

The cell concentration and viability data for this second HPCHO fed-batch experiment are shown in Figure 17. The culture experienced a lag phase until around 30 hours. During this time, the CHO cells were adjusting to the change in environment resulting from transitioning from the spinner flask to the stirred tank reactor. Interestingly, this period was shorter in this second fed-batch experiment than in the first fed-batch experiment. This probably occurred for three reasons. First, the initial cell concentration in the reactor was slightly higher in the second fed-batch experiment than in the first fed-batch experiment (1.75×10^5 cells/mL rather than 1.5×10^5 cells/mL). This concentration, however, was still below the initial cell concentration in the batch experiment of 2.5×10^5 cells/mL. Secondly, the working volume of the second fed-batch experiment was slightly less and this may have been a better fluid dynamic situation for the movement of the cells around the impeller blades of the reactor. Lastly, the addition of the concentrated essential amino acid solution to the feed medium provided a minimal amount of necessary amino acids to the CHO cells that the cells were not producing on their own. The concentration of one of these essential amino acids, other than glutamine, may have been a limiting factor in the growth and division of the cell culture. This difference in the length of the lag phase also caused the start and end of the exponential growth phase of the two fed-batch cultures to differ by about 15 hours. This also caused the start and end of the exponential growth phase of the second fed-batch culture to match that of the batch experiment. A graph showing the cell concentration data for both the batch and the second fed-batch CHO experiments is shown in Figure 18, so that the two experiments may be compared visually. The fed-batch data series have solid lines and the batch data series have dashed lines.

Exponential growth of the second fed-batch culture of HPCHO cells began around 30 hours and continued until around 80 hours. The average growth rate during this period was 0.045/hr. The maximum growth rate during this period was 0.055/hr and

occurred in the beginning of the exponential growth phase around 30 hours. The maximum growth rate of the exponential phase of the second fed-batch culture was comparable to that of both the batch and the first fed-batch culture. The average growth rate of the exponential phase of the second fed-batch culture, however, was significantly higher than that of both the batch and the first fed-batch culture. This situation is well illustrated by Figure 18. At 55 hours, the viable cell concentrations of the batch and second fed-batch cultures were approximately even. By 80 hours, however, the second fed-batch culture had grown rapidly to a viable cell concentration that is 20% higher than the concentration the batch culture had reached. The fed-batch culture experienced a quite short stationary phase from around 80 hours to about 85 hours. This is substantially shorter than the stationary phase of the first fed-batch, and is most easily explained by discussions of the metabolite concentrations, the cell diameter, and the osmolality of the medium below. Finally, the fed-batch experiment experienced a death phase that began around 85 hours and continued until about 145 hours, when the viability of the cells approached 35% and the experiment was ended.

The maximum viable cell concentration achieved by the second fed-batch CHO culture was 41.19×10^5 cells/mL at 80 hours. The maximum total cell concentration reached was 41.96×10^5 cells/mL, also at 80 hours. Although the maximum total cell concentration was much lower in the second fed-batch than in the batch experiment (the maximum total cell concentration was 50.72×10^5 cells/mL at 175 hours in the batch experiment), the maximum viable cell concentration was higher in the second fed-batch experiment than in either the batch experiment or the first fed-batch experiment (the maximum viable cell concentration was 38.62×10^5 cells/mL at 105 hours in the batch experiment and 31.37×10^5 cells/mL at 95 hours in the first fed-batch experiment). Therefore, if the productivity of the HPCHO cells were evaluated purely based on the maximum viable cell concentration achieved, the second fed-batch experiment that added concentrated essential amino acid solution to the feed would have produced more than both the batch and the first fed-batch runs. The viability of the second HPCHO fed-batch culture was quite high throughout the lag, exponential growth, and stationary phases. The viability of the cells remained above 95% until 95 hours and remained above 75%

until 105 hours. The cells in the second fed-batch culture began dying around 80 hours, shortly after the second bulk feed of nutrients.

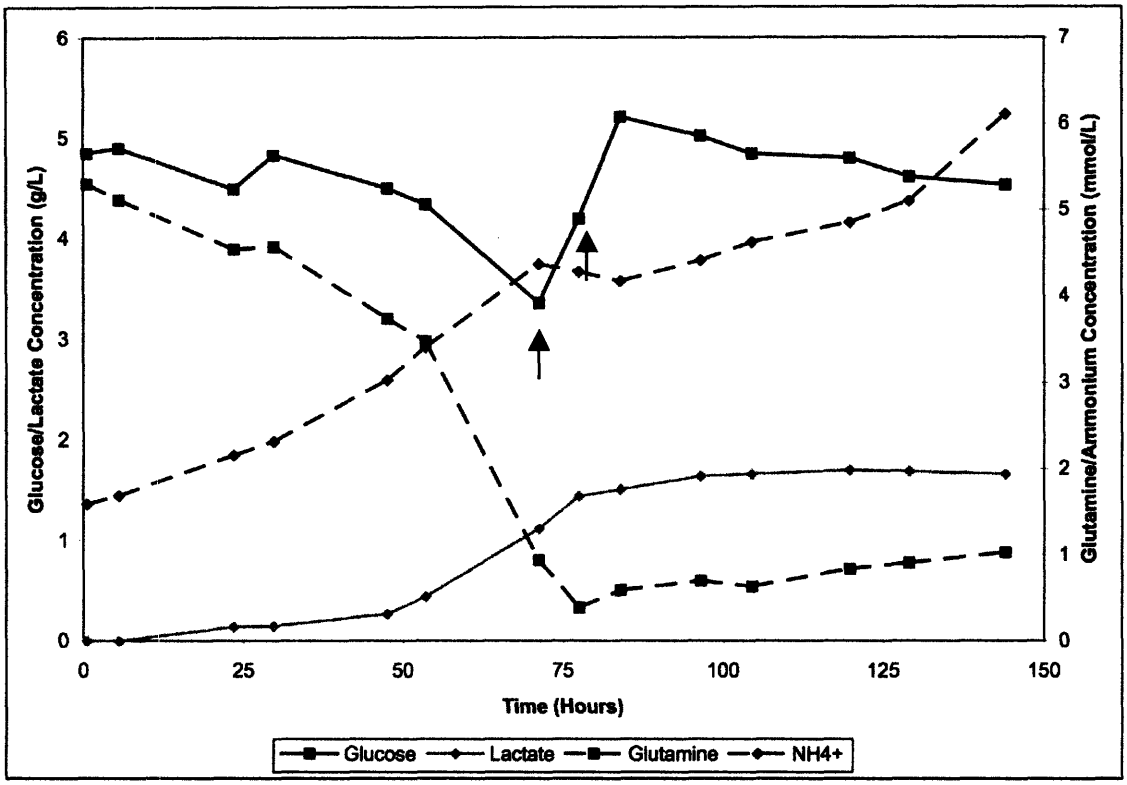

Figure 19 – Metabolic Data for HPCHO Fed-Batch Run 2. The black arrows indicate feeding doses.

The major metabolite concentrations of the second fed-batch HPCHO culture are shown in Figure 19. The initial glucose concentration was about 5 g/L. This value dropped slowly to about 3.25 g/L at around 70 hours, prior to the first feeding. The two bulk doses of nutrients then increased the glucose concentration to about 5.25 g/L at 85 hours. The glucose concentration then proceeded to slowly decrease to a final value of 4.5 g/L at 145 hours. Therefore, the HPCHO cells in the second fed-batch experiment used glucose at a much lower rate then the cells in the batch and previous fed-batch experiments. This observation is further substantiated by the trends in lactate concentration. The lactate concentration began at 0 g/L and increased slowly to about 1.5 g/L by 80 hours. After this point, the lactate concentration continued to increase quite slowly until it was around 1.7 g/L at 145 hours. The increases in lactate concentration corresponded to the decreases in glucose concentration. It is important to note that the amount of glucose taken up by the HPCHO cells in the second fed-batch experiment was

about the same as the amount of lactate produced by the cells. This is evidence that the cells continued to take up glucose slowly after the bulk doses of feeding and used the glucose to continue to grow and divide rather than produce. Further evidence of this observation lies in the cell diameter and osmolality data, which will be discussed below. The opposite trend was true for the first fed-batch experiment. In that experiment, the glucose was probably used by the HPCHO cells to produce more monoclonal antibody rather than to perform cell metabolism. This led to a disproportionately small concentration of lactate produced by the cells.

The trends of glutamine and ammonium ion concentration in the second HPCHO fed-batch culture were more complex than those of the batch and first fed-batch experiments. The initial glutamine concentration of the second HPCHO fed-batch culture was about 5.25 mmol/L and was reduced to under 0.5 mmol/L by 78 hours. There were two regions of glutamine consumption, however. Prior to 55 hours, the glutamine concentration decreased less quickly than between 55 and 78 hours. After 78 hours, the glutamine concentration slowly increased to around 1 mmol/L. This increase was probably due to conversion of some of the essential amino acids to glutamine. The concentration of ammonium ion began at around 1.5 mmol/L and increased steadily to over 4 mmol/L at 78 hours. The ammonium ion concentration then continued to increase at a slower rate to over 6 mmol/L at 145 hours, most likely due to death of the cells. Overall, the concentration of ammonium ion was less in the second fed-batch than in the first fed-batch. The cells were able to take up more nutrients in the second fed-batch for the purpose of growing and dividing (29). It appears that the culture utilized glutamine at a faster rate than it used glucose and produced ammonium ion at a faster rate than it created lactate in the beginning of the culture, when the cells were multiplying and dividing. This observation would be incorrect, however, since less glutamine then glucose was initially available to the culture. Once the feed medium was supplied to the culture, the CHO cells began using primarily glucose and the concentration of glutamine increased due to the conversion of essential amino acids.

The diameter of the HPCHO cells and the osmolality of the medium in both the first fed-batch and the second fed-batch cultures are shown in Figure 20. The bulk doses of feed medium delivered to the second fed-batch culture of HPCHO cells produced

sharp increases in the osmolality of the culture. After the first feeding at around 72 hours, the osmolality increased from about 330 mOsm/kg to about 400 mOsm/kg. After the second feeding at around 78 hours, the osmolality increased from around 400 mOsm/kg to nearly 460 mOsm/kg. The highest osmolality reached in the second fed-batch experiment was 40 mOsm/kg less than that reached in the first fed-batch experiment. After the 105 hour time point, the osmolality of the culture was steady. The increased osmolality of the medium corresponded to the death phase of the second fed-batch culture, when the cells were no longer dividing, but were dying. From Figure 20, it is also apparent that this increase in osmolality actually corresponded to a decrease in average cell diameter of the cells in the second fed-batch culture. The average cell diameter was around 16 microns during the exponential growth phase of the culture. After 78 hours, the average cell diameter decreased steadily to about 14 microns at 105 hours. The average cell diameter then increased again to about 16 microns by 145 hours. This is the opposite result than the one seen in the first fed-batch experiment.

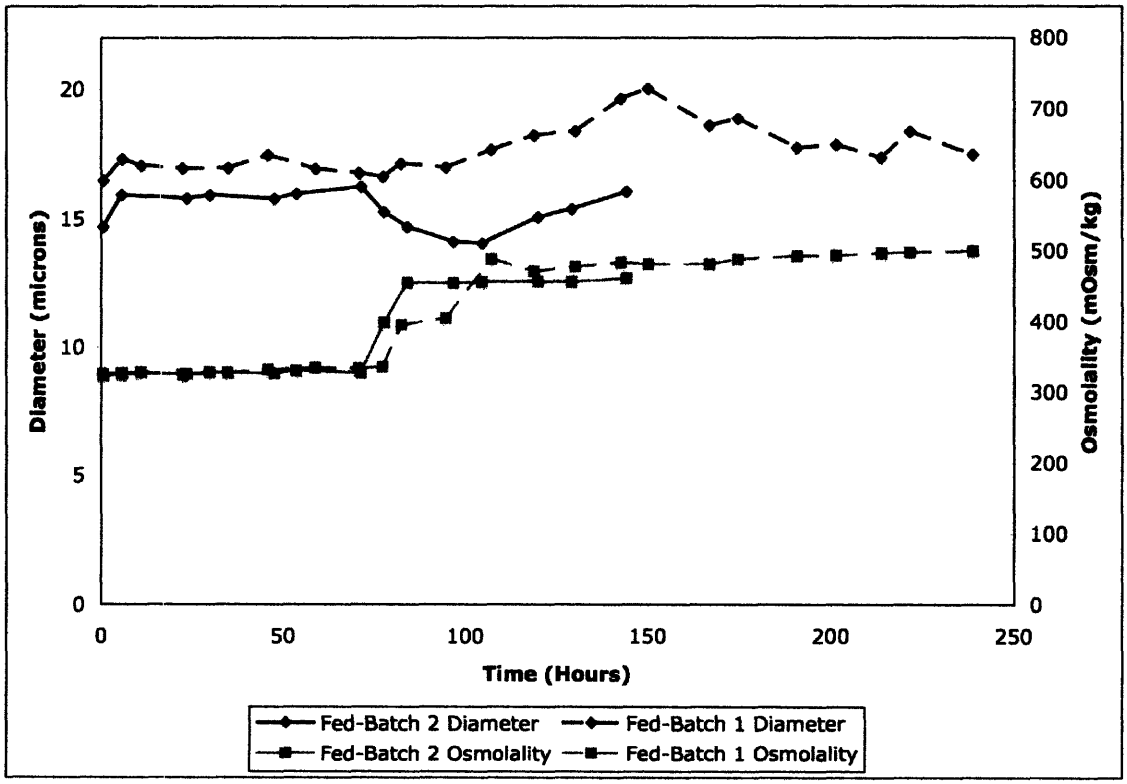

Figure 20 – Comparison of Diameter and Osmolality Data for HPCHO Fed-Batch Run No. 1 and HPCHO Fed-Batch Run No. 2

The possibility that the osmolality of the medium and average cell diameter may indicate the productivity of a culture was discussed previously in the section describing the HPCHO batch experiment. Since the osmolality increase was less drastic in the second fed-batch experiment, it can be assumed that the cells produced less monoclonal antibody per cell than in the first fed-batch experiment. This observation is confirmed by the fact that the cells in the second fed-batch experiment did not increase significantly in diameter (in fact, the cells decreased in diameter), but the cells in the first fed-batch experiment did increase significantly in diameter. Since the second fed-batch culture produced a higher maximum viable cell density but the first fed-batch culture contained more productive cells, it is difficult to determine which fed-batch culture produced the most monoclonal antibody. It is apparent, however, that the HPCHO cells in both of the fed-batch experiments produced more monoclonal antibody than HPCHO cells in the batch experiment.

The fed-batch experiments with HPCHO cells described above highlight an important issue in the design of feeding strategies. The different nutrients in the cell culture medium may be used for different purposes and the concentrations of waste products are also quite important. In the batch experiment and the second fed-batch experiment, the glutamine taken up by the cells caused a surge in cell density. Although the use of glutamine by the HPCHO cells produced ammonium ion, which can hinder glucose uptake (29), the levels of the toxic ion were low enough to allow the remaining glucose (or in the case of the second fed-batch, the added glucose of the feed medium) to be taken up by the cells for the purpose of growing and dividing. In the first fed-batch experiment, the glutamine taken up by the cells also caused a surge in cell density. However, the amount of ammonium ion produced by the use of glutamine was much higher than in the other two experiments. The HPCHO cells then slowly took up glucose for the purpose of producing monoclonal antibody rather than growing and dividing. The lower concentrations of lactate in the first fed-batch experiment support this observation. The average cell diameter and osmolality data for all three experiments are further evidence.

An ideal fed-batch medium for the HPCHO Chinese hamster ovary cell line would minimize glutamine and the production of ammonium ion, so that more glucose

could be taken up by the cells for the dual purposes of growing and producing monoclonal antibody. Another method to create an optimal fed-batch system with the HPCHO cells would involve a biphasic procedure, much like the method used to produce erythropoietin from a recombinant CHO cell line (31). In the initial batch phase of the process, a low osmolality medium would be used to grow the HPCHO cells to a relatively high concentration. A high osmolality medium would be used as the fed-batch medium. Feeding of this medium would cause osmotic stress to the cells and induce enhanced production of monoclonal antibody.

3.3 Ceramic membrane system

Several experiments involving the stirred ceramic membrane reactor (SCMR) system were performed with the HPCHO Chinese hamster ovary cell line as described above. A control experiment was first performed with the HPCHO Chinese hamster ovary cell line to evaluate the extent that the ceramic membrane module affected the culture environment of the bioreactor. In this experiment, the stirred ceramic membrane module was placed inside the three-liter bioreactor, but no feeding and harvesting of medium was initiated. The three-liter bioreactor was inoculated with a 1.8-liter working volume of HPCHO culture with an initial density of around 1.8×10^5 cells/mL. The only disturbances to the culture included the purging of the sample line of 3 mL and the sampling of 5 mL twice daily. The total amount of medium supplied to the HPCHO control batch experiment was 1.8 liters.

The cell concentration and viability data for this control SCMR experiment with the HPCHO cell line are shown in Figure 21. The graph shows no major differences between the HPCHO batch experiment and this experiment that included the ceramic membrane module inside the bioreactor. The phases of cell growth are of similar lengths for the cultures in both experiments, although the cells in the culture containing the ceramic membrane module died more quickly than the cells in the culture with no ceramic membrane module. The maximum viable cell concentrations and maximum total cell concentrations of the two experiments were quite similar. There were also no striking differences in the metabolite concentrations of the two experiments. If

productivity were qualitatively determined based either on viable cell concentration or osmolality changes in the culture medium, there would also be no significant difference in the monoclonal antibody productivity of the batch containing the ceramic membrane module and the batch not containing the ceramic membrane module. It was concluded that the inclusion of the ceramic membrane module in the bioreactor had little to no adverse effect on the HPCHO cell culture.

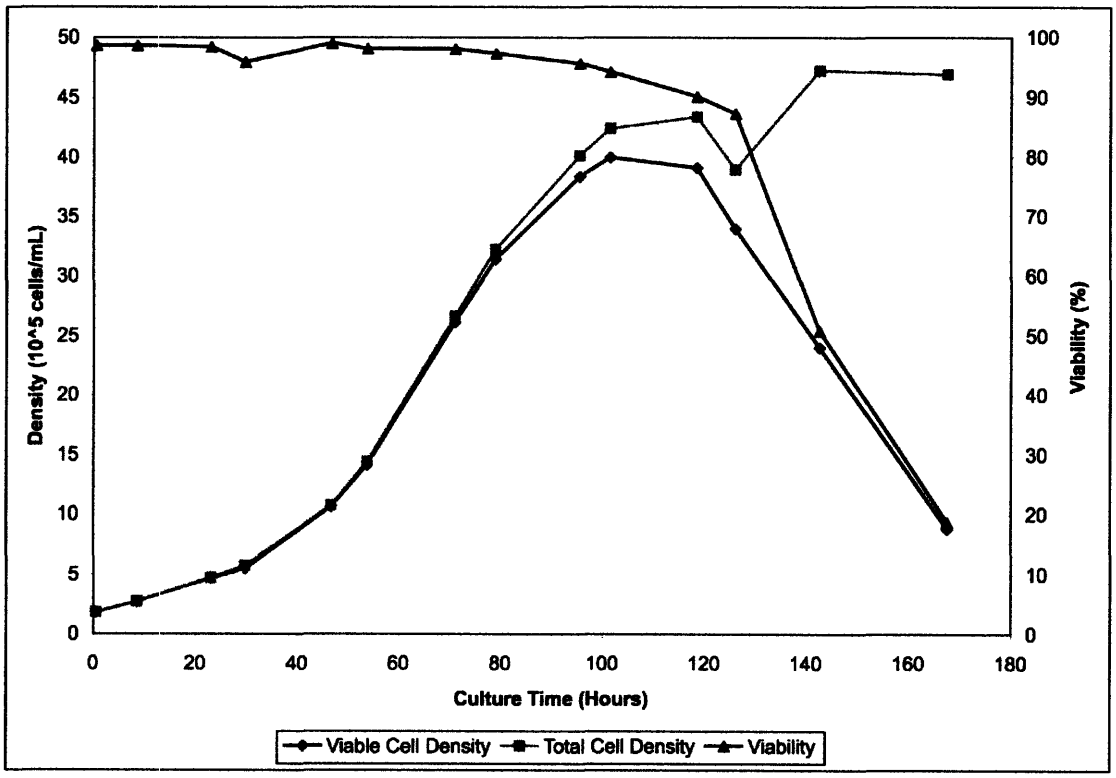

Figure 21 – Growth Data for HPCHO SCMR Control Batch Run

Perfusion experiments were next performed to evaluate the use of the SCMR system with the HPCHO cell line. The optimal perfusion feeding strategy was determined partly from previous research with this equipment (23-25) and partly from repeated experiments testing the equipment and feeding strategies on the HPCHO cells. The optimal set of feeding rates found by previous research (24) for perfusion culture of IB4 hybridoma cells in serum-free medium in a 1 liter working volume are shown in Table 2. The optimal set of feeding rates found for SCMR perfusion culture of HPCHO Chinese hamster ovary cells in a 50-50 mixture of BD CHO Medium (BD Biosciences, MD) and Sigma-Aldrich Ex-Cell Animal Component Free CHO Medium (Sigma-Aldrich Corp., MO) are shown in Table 3. The two sets of perfusion rates do not match. It is

important to note that every attempt was made to increase the perfusion rate at set stages, but due to fouling of the ceramic membrane module, the perfusion rates were often lower than what had been expected.

Table 2 – Optimal Set of Perfusion Rates for SCMR Culture of IB4 Hybridoma Cells in Serum-Free Medium in a 1 Liter Working Volume (adapted from 24).

Time	Perfusion Rate (Volume of Fresh Medium / Working volume / Day)
0 – 55 hours	0 vvd
55 – 73 hours	1 vvd
73 – 117 hours	Increased rapidly from 1 – 3 vvd
117 – 260 hours	3 vvd

The most successful SCMR experiment with the HPCHO Chinese hamster ovary cell line will be discussed. The three-liter bioreactor was inoculated with a 1.8-liter working volume of HPCHO culture with an initial density of around 2.4 x 10^5 cells/mL. Disturbances included the purging of the sample line of 3 mL and the sampling of 5 mL twice daily, as well as feeding of fresh medium and harvesting of spent medium after 70 hours. The autoclaved 3 liter bottles that contained the fresh medium to be fed and that collected the spent medium were replaced with a new set of autoclaved bottles after 163 hours. The total amount of medium supplied to the HPCHO SCMR experiment was 9.8 liters.

Table 3 – Actual Set of Perfusion Rates for SCMR Culture of HPCHO Cells

Time	Perfusion Rate (Volume of Fresh Medium / Working volume / Day)
0 – 70 hours	0 vvd
70 – 104 hours	1.1 vvd
104 – 175 hours	1.4 vvd

The cell concentration and viability data for the HPCHO SCMR experiment are shown in Figure 22. A graph comparing the cell concentration and viability data for both the batch and the SCMR CHO experiments is shown in Figure 23, so that the two experiments may be compared visually. The SCMR data series have solid lines and the batch data series have dashed lines. The SCMR culture experienced a lag phase until

around 30 hours. The lag phase was due to the adjustment of the CHO cells to the new environment of the stirred tank reactor. Because this period was similar in length to the lag phase in the batch experiment, the observation that the presence of the ceramic membrane module had no significant adverse effect on the culture environment was further verified.

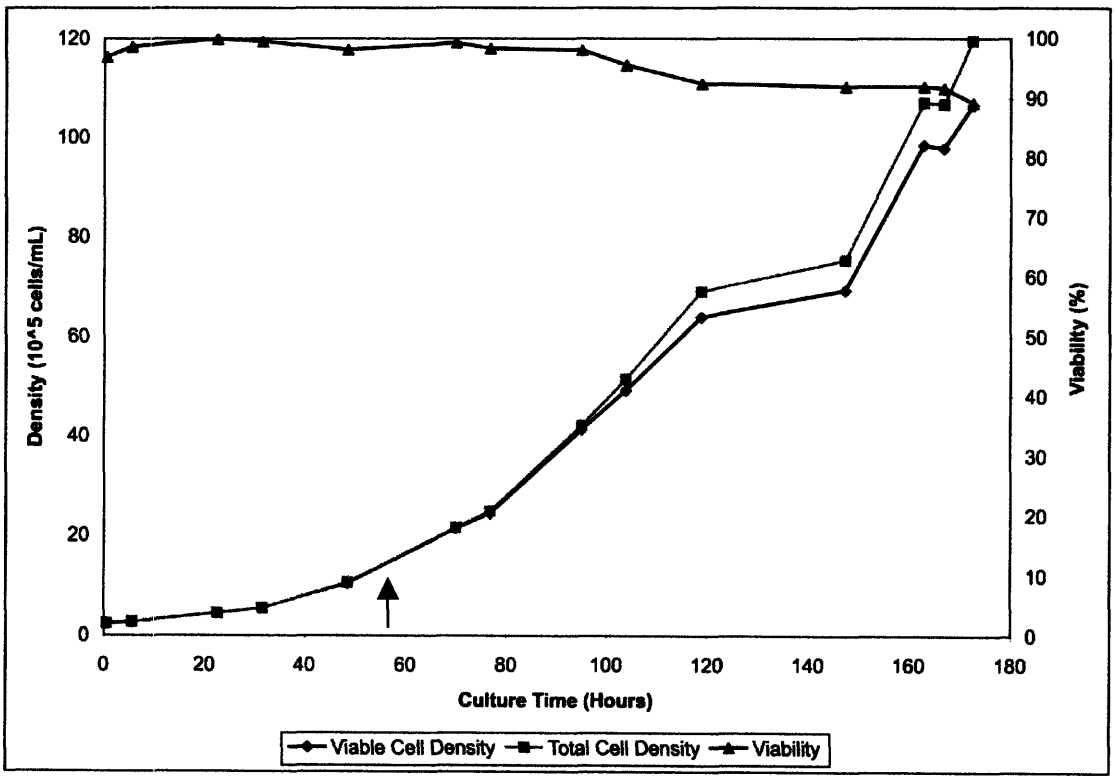

Figure 22 – Growth Data for HPCHO SCMR Run. The black arrow indicates the start of perfusion.

There were three phases of exponential growth of the SCMR culture. The first exponential growth phase began around 30 hours and continued until about 77 hours. This time period corresponds to the growth possible in batch conditions. The average growth rate during this period was 0.031/hr. The maximum growth rate during this period was 0.038/hr at about 50 hours. The average growth rate of this time period of the SCMR culture was slightly faster than that for the batch HPCHO culture, which was 0.026/hr. Feeding and harvesting of the bioreactor were initiated after 70 hours. The second exponential growth phase began around 77 hours and continued until 120 hours. This time period corresponds to extended growth of the culture due to perfusion. The average growth rate during this period was 0.021/hr, which is slightly slower than that for the batch HPCHO culture. The third exponential growth phase began around 120 hours

and continued until the end of the culture at about 175 hours. This time period corresponded to continued growth of the culture, but at a slowed pace. Significant aggregation of the HPCHO cells occurred and clumps of cells were visible in the bioreactor during this third exponential growth phase. The average growth rate during this period was 0.0096/hr. This average growth rate was much slower than that of the batch HPCHO culture. This was most likely due to a decrease in nutrients that were available to the high density of CHO cells. The perfusion rates shown in Table 3 are evidence of this theory. Although the rotation of the peristaltic pump was increased twice during the run in an attempt to increase the perfusion rates of the SCMR experiment, the final perfusion rate was quite similar to the previous perfusion rates due to increased fouling of the ceramic membrane module. This lower than desired perfusion rate allowed less nutrients to reach the high density of HPCHO cells than was desired. The various metabolite concentrations of the SCMR experiment will be examined in more detail below to verify these observations. Due to the aggregation of CHO cells and the extensive fouling of the ceramic membrane module, the experiment was halted at about 175 hours when medium was no longer harvested from the ceramic membrane module. Consequently, the SCMR culture experienced no stationary phase and no death phase.

The maximum viable cell concentration reached in the HPCHO SCMR experiment was 106.6 x 10^5 cells/mL at about 175 hours, the end of the culture. As Figure 23 illustrates, this value was over 2.5 times that achieved in the HPCHO batch experiment. If the productivity of the HPCHO culture were qualitatively determined strictly on the basis of viable cell concentration as discussed previously, the SCMR culture would have produced over 2.5 times more IgG_1 antibody than the batch culture. The maximum total cell concentration achieved was 119.5 x 10^5 cells/mL, also at the end of the culture at 175 hours. As Figure 23 shows, this value was over twice that achieved in the HPCHO batch experiment. The viability of the SCMR culture remained high throughout the experiment. The viability of the cells remained above 95% until after 105 hours and above 90% until the very end of the experiment. This showed that perfusion involving the ceramic membrane module had little to no adverse effect on the viability of the HPCHO cells. Taken together, this data showed that the feeding of fresh medium and

the harvesting of spent medium allowed the HPCHO culture to reach much higher cell concentrations while maintaining a relatively high viability of the culture and producing a significantly greater amount of monoclonal antibody than the batch experiment.

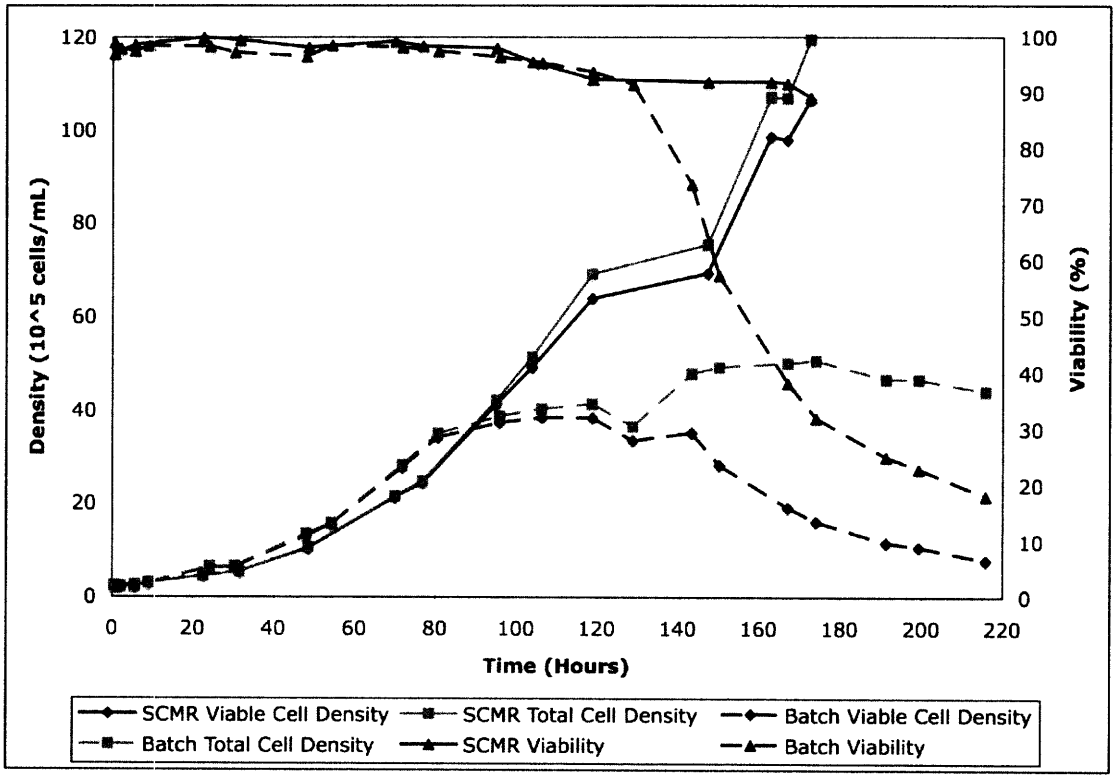

Figure 23 – Comparison of Growth Data for HPCHO Batch Run and HPCHO SCMR Run

Major metabolite concentrations of the HPCHO SCMR culture are illustrated in Figure 24. At inoculation, the glucose concentration was about 5.5 g/L. The glucose concentration decreased steadily until about 70 hours when it was about 3 g/L. Perfusion of the SCMR system was initiated at this point. After 70 hours, the glucose concentration increased slightly to nearly 3.5 g/L and leveled off at this value for about 30 hours. The glucose concentration then began decreasing steadily again until it was nearly exhausted at 160 hours. This decrease in nutrients occurred because the perfusion rates were less than expected and not enough glucose was added to the culture to replenish the metabolites taken up by the high density of CHO cells. At inoculation, the concentration of lactate in the culture was 0 g/L. During the batch phase of the experiment, the concentration of lactate climbed steadily as the glucose in the culture was consumed. At 70 hours, the concentration of lactate was about 1.8 g/L. Most of the glucose consumed by the culture to this point, therefore, was used for cellular metabolism and growth, rather

than production of monoclonal antibody. After perfusion of the SCMR system was initiated, the concentration of lactate in the culture decreased slightly and leveled off at a value of 1.5 g/L for 30 hours. The ceramic membrane module was able to remove some of the lactate from the culture, but not enough to keep the concentration of lactate constant for longer than 30 hours. At 100 hours, the lactate concentration began rapidly increasing to a final value of over 5.5 g/L at the end of the culture. Again, the majority of the glucose was apparently converted into lactate, but some of the glucose added to the culture during the perfusion phase may have been used to produce the IgG_1 monoclonal antibody.

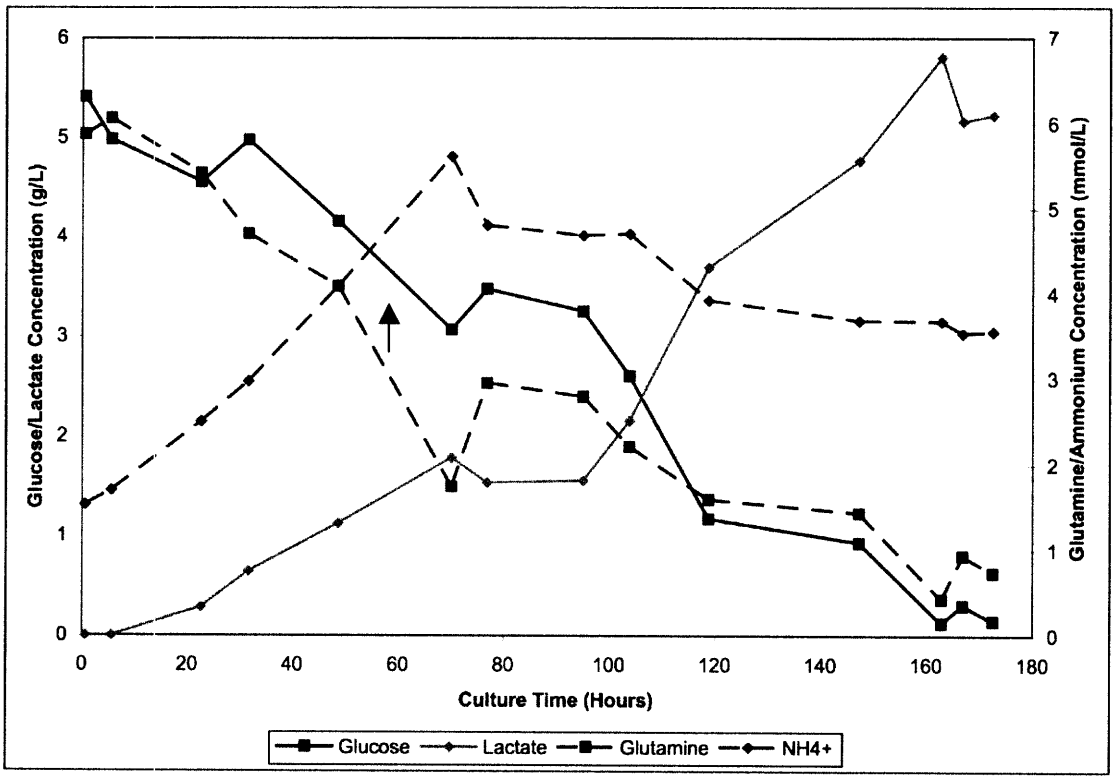

Figure 24 -- Metabolite Data for HPCHO SCMR Run. The black arrow indicates the start of perfusion.

At inoculation, the glutamine concentration in the HPCHO SCMR experiment was about 6 mmol/L. The concentration of this metabolite decreased steadily until it reached 1.75 mmol/L at 70 hours. The perfusion of fresh medium was initiated at this time point. After perfusion was initiated, the glutamine concentration increased to nearly 3 mmol/L by 77 hours. The glutamine concentration remained steady for almost 20 hours, but then began decreasing after 95 hours. This was because the very high density

of CHO cells was utilizing the glutamine before it could be fully replenished by perfusion at the lower than desired rates. By 160 hours, the glutamine concentration was about 0.5 mmol/L. After this point, the glutamine concentration increased slightly to nearly 1 mmol/L. This increase also corresponded to the largest decrease in cell viability. Cells in the culture were beginning to die and the filter was beginning to foul significantly, so the concentration of all nutrients increased slightly. At inoculation, the concentration of ammonium ion in the culture was about 1.5 mmol/L. The ammonium ion concentration increased steadily during the batch phase of the experiment as the glutamine concentration decreased. At 70 hours, the concentration of ammonium ion in the culture was over 5.5 mmol/L. After perfusion was initiated, the concentration of ammonium ion decreased and leveled off to a final value of around 3.5 mmol/L at the end of the culture. This trend showed that the ceramic membrane module was effective in harvesting ammonium ion, a toxic waste product that can inhibit the uptake of glucose by CHO cells, from the reactor and maintained the concentration of that toxic waste product at a relatively low value.

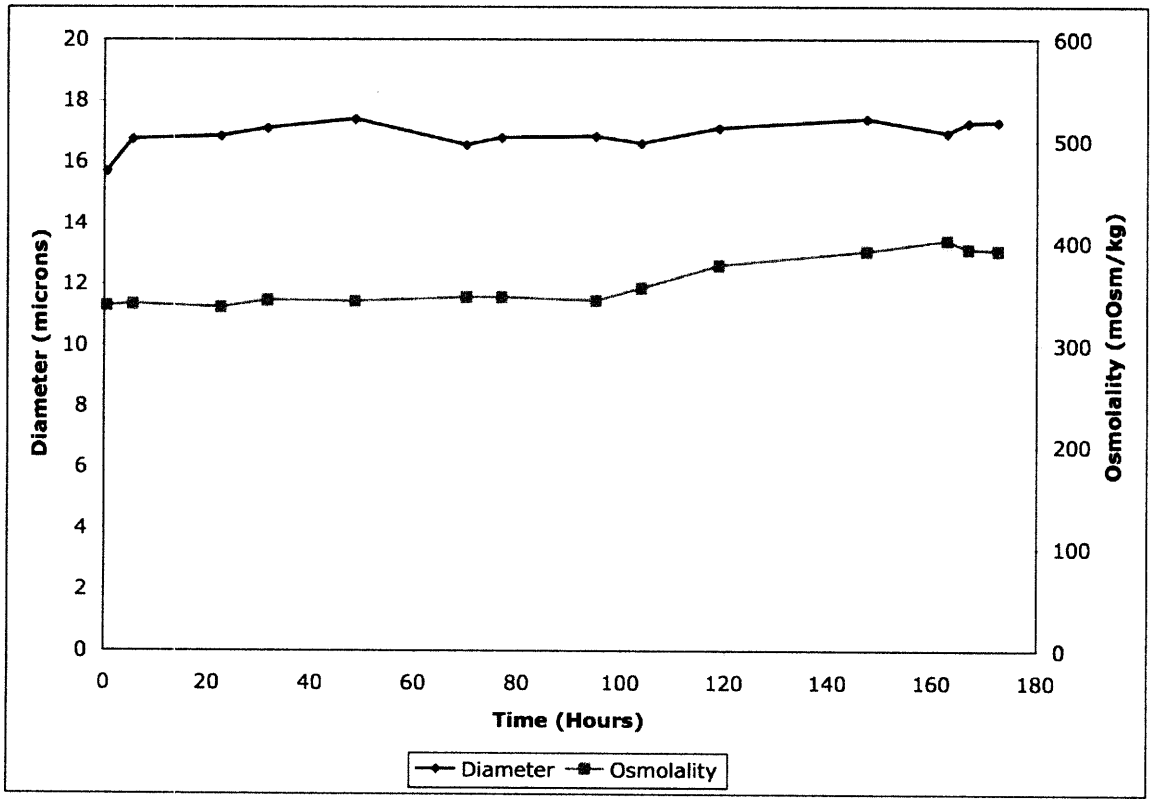

Figure 25 – Diameter and Osmolality Data for HPCHO SCMR Run

The theory that cell diameter and culture osmolality trends could provide insight into the monoclonal antibody productivity of CHO cells was discussed previously. The diameter and osmolality data for the HPCHO SCMR experiment are shown in Figure 25. The graph shows little change in cell diameter. Throughout the experiment, the average cell diameter ranged from 16.5 microns to 17.5 microns. The graph also shows only a slight change in the osmolality of the culture medium. At inoculation, the osmolality of the medium was about 340 mOsm/kg. After 100 hours, the osmolality of the medium began increasing slightly. The osmolality then leveled off at below 400 mOsm/kg by the end of the experiment.

These trends were quite similar to that observed in the batch HPCHO experiment and highlight two important characteristics of the culture. First, it is apparent that the ceramic membrane module is quite effective in removing ions from the bioreactor that may cause stress to the cells and cause the cell diameter to increase. Second, the individual cells in the SCMR culture were producing less monoclonal antibody than the individual cells in the first fed-batch culture of HPCHO Chinese hamster ovary cells. However, the individual cells in the SCMR culture were likely producing about the same amount of monoclonal antibody as the individual cells in the batch culture of HPCHO cells. Because the maximum viable cell concentration reached in the SCMR culture was much higher than any previous experiment involving the other feeding strategies, it can be assumed that the SCMR run produced more monoclonal antibody than the batch or fed-batch runs. It is important to note, however, that the IgG_1 monoclonal antibody produced by the HPCHO cells in perfusion runs may be more degraded than the product from experiments involving other feeding strategies. Differences in glycosylation of monoclonal antibodies have been observed in cultures grown by different feeding strategies (33).

3.4 Alternating tangential flow hollow fiber system

Several perfusion experiments involving the alternating tangential flow (ATF) hollow fiber membrane system were performed with the HPCHO Chinese hamster ovary cell line as described above. The optimal settings for the ATF controller were

determined from previous unpublished research (34) and some experimentation. The ideal pressure set point of the ATF controller was about 4 psi and the exhaust cycle lasted approximately 10 seconds. The optimal perfusion feeding strategy was determined partly from previous research with other equipment such as the SCMR system and partly from repeated experiments testing the ATF equipment and feeding strategies on the HPCHO cells. The optimal set of feeding rates found for ATF perfusion culture of HPCHO Chinese hamster ovary cells in a 50-50 mixture of BD CHO Medium (BD Biosciences, MD) and Sigma-Aldrich Ex-Cell Animal Component Free CHO Medium (Sigma-Aldrich Corp., MO) are shown in Table 4. The perfusion rates for the ATF experiment do not exactly match the perfusion rates for the SCMR experiment. It is important to note that every attempt was made to significantly increase the perfusion rate at set stages, but due to fouling of the hollow fiber membrane, the perfusion rates were often lower than what had been expected. After 150 hours, there were also some problems with keeping the working volume in the bioreactor constant. At times, 200 mL of extra medium was fed to the reactor, and at other times, an extra 200 mL of extra medium was fed to the reactor. The perfusion rates in table 4 and the other data presented below represent the best estimate of the actual conditions in the bioreactor.

Table 4 – Actual Set of Perfusion Rates for ATF Culture of HPCHO Cells

Time	Perfusion Rate (Volume of Fresh Medium / Working volume / Day)
0 – 70 hours	0 vvd
70 – 95 hours	1 vvd
95 – 119 hours	1.3 vvd
119 – 142 hours	1.5 vvd
142 – 220 hours	1.7 vvd

The most successful ATF experiment with the HPCHO Chinese hamster ovary cell line will be discussed. The three-liter bioreactor was inoculated with a 1.8-liter working volume of HPCHO culture with an initial density of around 2 x 10^5 cells/mL. Disturbances included the purging of the sample line of 3 mL and the sampling of 5 mL twice daily, as well as feeding of fresh medium and harvesting of spent medium after 72 hours. The autoclaved 3 liter bottles that contained the fresh medium to be fed and that collected the spent medium were replaced with a new set of autoclaved bottles after 106

hours, 130 hours, 154 hours, and 168 hours. The total amount of medium supplied to the HPCHO ATF experiment was 19.6 liters.

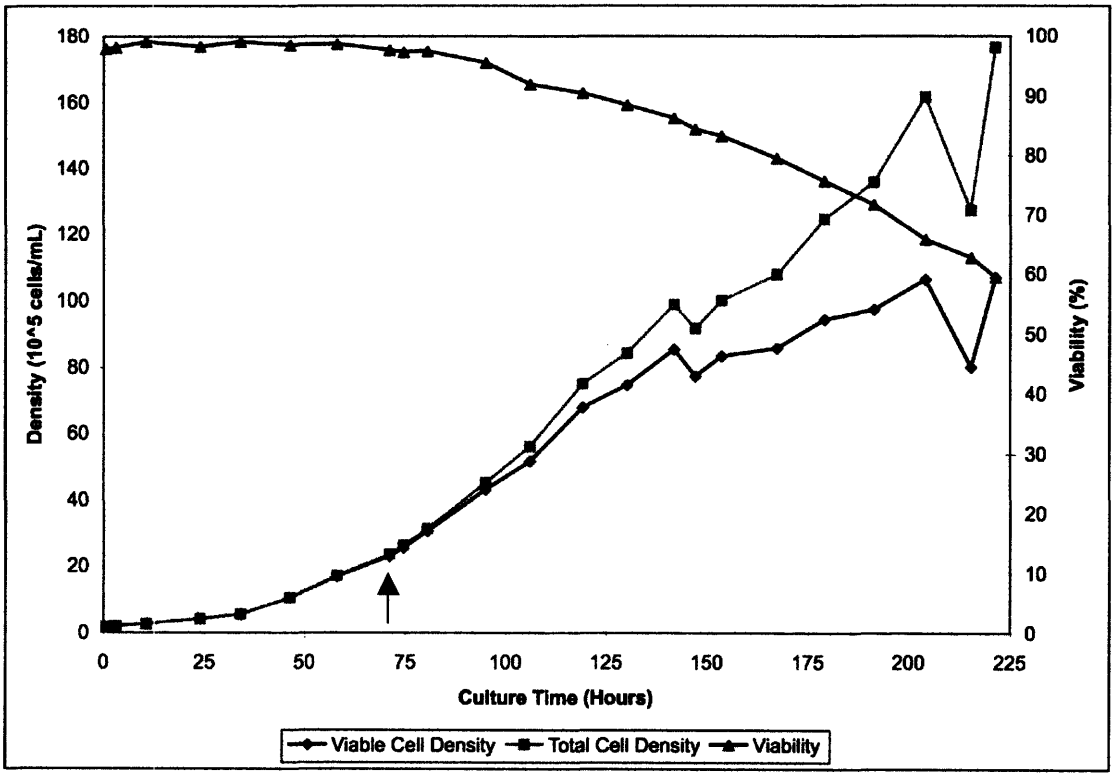

Figure 26 – Growth Data for HPCHO ATF Run. The black arrow indicates the start of perfusion.

The cell concentration and viability data for the HPCHO ATF experiment are shown in Figure 26. A graph illustrating the cell concentration and viability data for both the batch and the ATF CHO experiments is shown in Figure 27, so that the two experiments may be compared visually. The ATF data series have solid lines and the batch data series have dashed lines. Figure 28 contains a graph comparing the cell concentration and viability data for the ATF and SCMR HPCHO experiments. The ATF data series again have solid lines and the SCMR data series have dashed lines. The ATF culture experienced a lag phase until around 30 hours. The lag phase was due to the adjustment of the CHO cells to the new environment of the stirred tank reactor. Because this period was similar in length to the lag phases in both the batch and SCMR experiments, it was concluded that the ATF equipment set-up had little to no initial effect on the growth and viability of the HPCHO cells. This was to be expected since the ATF system is primarily an external perfusion system. During the later stages of the

experiment, however, the ATF experimental design would have an effect on the growth and viability of the HPCHO cells.

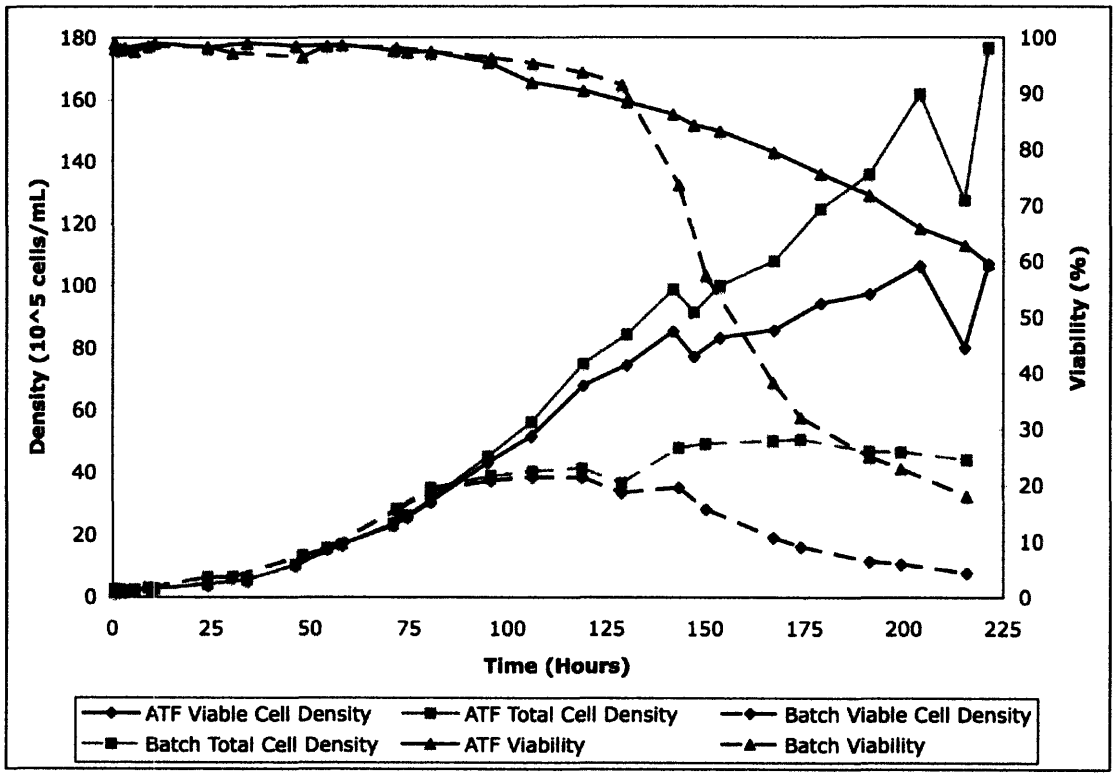

Figure 27 – Comparison of Growth Data for HPCHO Batch Run and HPCHO ATF Run

There were two different phases of exponential growth of the Chinese hamster ovary cell ATF culture. The first exponential growth phase began around 30 hours and continued until about 105 hours. This time period corresponds to the growth possible in batch conditions and the initial enhancement of cell growth created by the ATF system cycling. The average growth rate during this period was 0.033/hr. The maximum growth rate during this period was 0.051/hr at about 40 hours. The average growth rate of this time period of the ATF culture was slightly faster than that for the SCMR culture, which was 0.031/hr., and even faster than the average exponential growth rate of this time period for the batch HPCHO culture, which was 0.026/hr. Feeding and harvesting of the bioreactor were initiated after 72 hours. The second exponential growth phase began around 105 hours and continued until the conclusion of the ATF culture at around 220 hours. This time period corresponds to extended growth of the culture due to perfusion, though at a much reduced pace. The average growth rate during this period was 0.008/hr, which was substantially slower than all the growth rates during both the SCMR culture

and the batch HPCHO culture. This decrease in exponential growth rate was most likely due to two factors. First, the actual perfusion rates run during the ATF experiment were lower than what had been anticipated. The amount of nutrients available to the high density of Chinese hamster ovary cells was less than expected. Second, the ATF cycling began to affect the viability of the cells during this second exponential growth phase. The cells were not growing at as fast a rate as earlier in the experiment because many of the cells were dying due to shear forces generated by the ATF cycling. If the experiment had continued, the second exponential growth period would have probably become a stationary phase. However, there was significant aggregation of Chinese hamster ovary cells and the experiment was halted at 220 hours due to fouling of the hollow fiber membrane cartridge. Consequently, the HPCHO ATF culture experienced neither a true stationary phase nor a death phase.

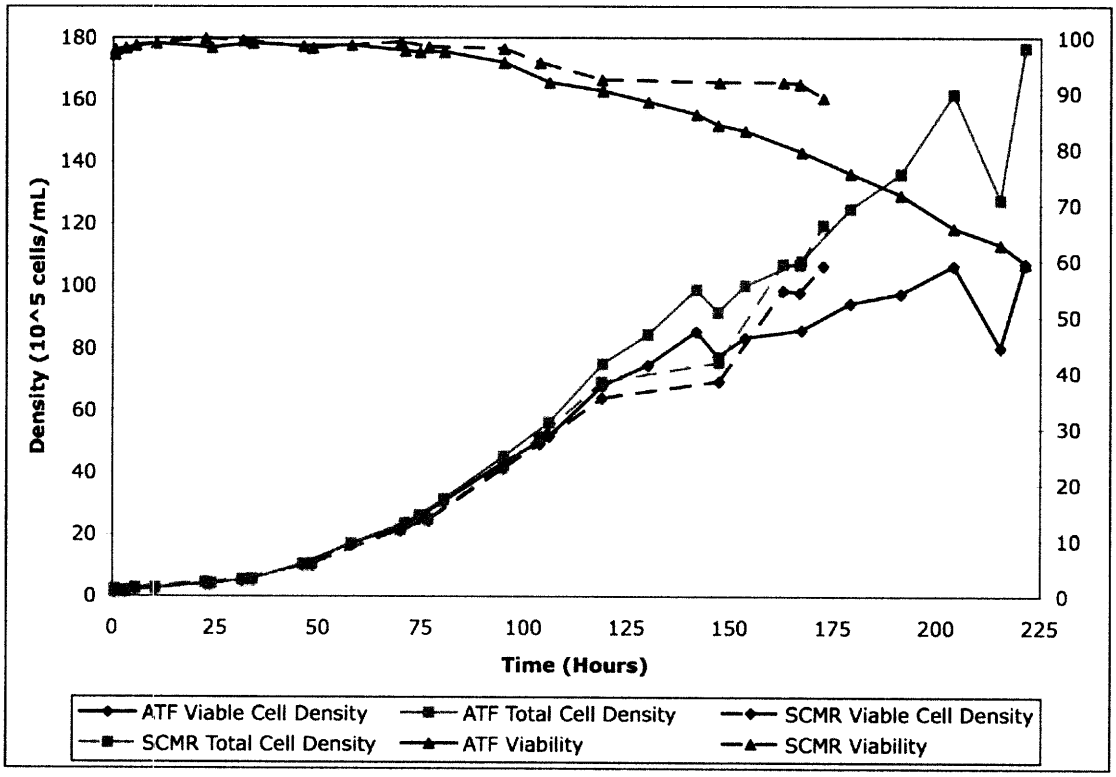

Figure 28 – Comparison of Growth Data for HPCHO SCMR Run and HPCHO ATF Run

The maximum viable cell concentration reached in the HPCHO ATF experiment was 107.16 x 10^5 cells/mL at about 220 hours, the end of the culture. As Figure 27 illustrates, this value was over 2.5 times that achieved in the HPCHO batch experiment. This value was also quite similar to the maximum viable cell concentration achieved in

the HPCHO SCMR (106.6 x 10^5 cells/mL). This is clearly shown in Figure 28. If the productivity of the HPCHO culture were qualitatively determined strictly on the basis of viable cell concentration as discussed previously, therefore, the ATF culture would have produced the same amount of IgG_1 antibody as the SCMR culture and both of these perfusion experiments would have produced over 2.5 times more IgG_1 antibody than the batch culture. The maximum total cell concentration achieved in the ATF experiment was176.67 x 10^5 cells/mL, also at the end of the culture at 220 hours. As Figure 27 shows, this value was over three times that achieved in the HPCHO batch experiment. This value was also much higher than the maximum total cell concentration achieved in the SCMR experiment (119.5 x 10^5 cells/mL), as illustrated in Figure 28. Although the maximum viable cell concentrations of the two perfusion experiments were similar, the ATF culture contained many more total cells than the SCMR culture.

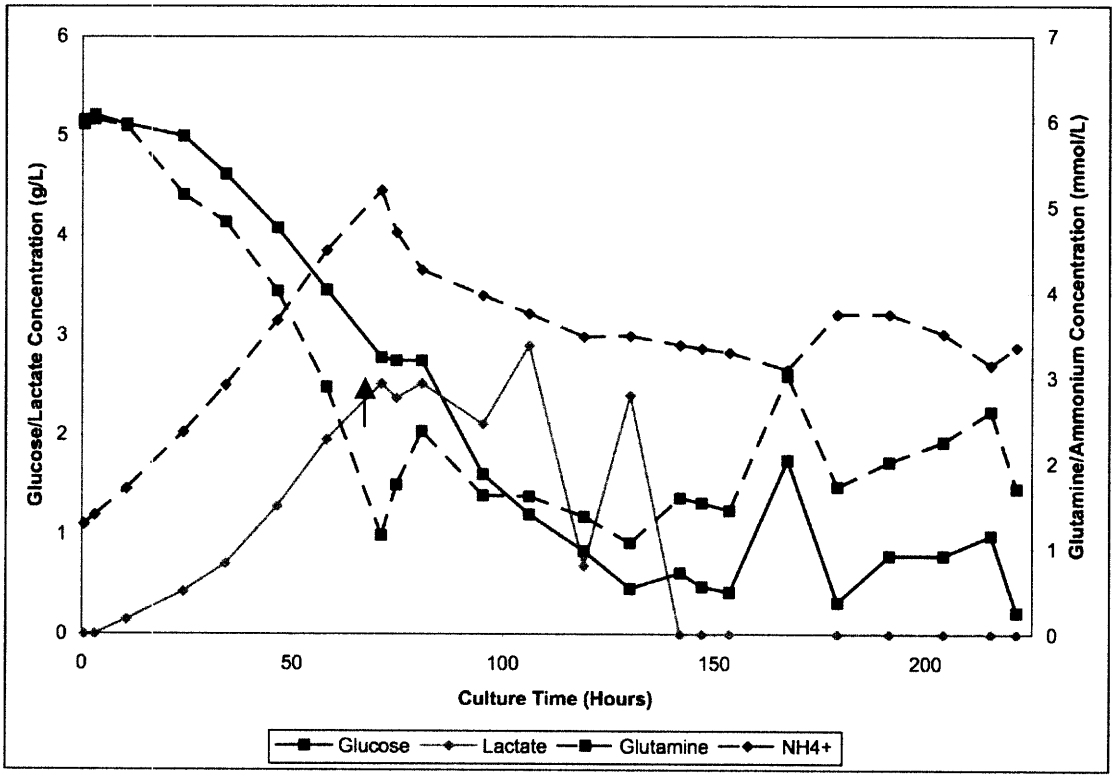

Figure 29 – Metabolic Data for HPCHO ATF Run. The black arrow indicates the start of perfusion.

Viability apparently became an issue during the ATF experiment. The viability of the HPCHO cells remained above 95% until around 95 hours and above 90% about 120 hours. After 120 hours, the viability of the cells steadily declined until they reached 60% viability at the end of the experiment. This showed that perfusion involving the ATF

hollow fiber membrane system had some effect on the viability of the HPCHO cells. Taken together, the data showed that the feeding of fresh medium and the harvesting of spent medium during the ATF experiment allowed the HPCHO culture to reach much higher cell concentrations and produce a greater amount of monoclonal antibody than the batch experiment, however, the impact of the ATF cycling on the viability of the Chinese hamster ovary cells was significant.

Major metabolite concentrations of the HPCHO ATF culture are illustrated in Figure 29. At inoculation, the glucose concentration was almost 5.2 g/L. The concentration of this metabolite decreased steadily until it reached about 2.75 g/L at 72 hours. Perfusion of the ATF culture was initiated at this point. The glucose concentration remained steady due to the addition of fresh medium and removal of spent medium for about 10 hours. After the Chinese hamster ovary cell culture had been growing for 80 hours, the perfusion rate was not sufficiently high to fully maintain the concentration of glucose in the reactor. The concentration of this metabolite slowly fell to less than 0.5 g/L after 150 hours. Around this time, the perfusion rate was increased to its highest value (1.7 vvd) and the viability of the culture was decreasing significantly. The concentration of glucose thus rose sharply to 1.75 g/L at 168 hours. The concentration of glucose then began to decline until it was again below 0.5 g/L at the end of the experiment. At inoculation, there was no lactate in the medium of the HPCHO culture. As glucose was consumed by the CHO cells, the lactate concentration increased quickly to slightly over 2.5 g/L. at 72 hours. Since this value is nearly equal to the amount of glucose consumed by the culture, it can be assumed that the HPCHO cells were mainly utilizing glucose for growth and cellular metabolism up to the point at which perfusion was initiated. The hollow fiber membrane cartridge was quite effective at removing lactate from the ATF culture. Consequently, the concentration of lactate was maintained at an average value of 2.5 g/L over the next 50 hours of the culture (there was some oscillation in concentration due to alterations in perfusion rate of the system). After 130 hours, all lactate was removed from the system and the concentration of this waste product remained at 0 mmol/L until the end of the culture. The lactate value at 168 hours was not considered due to a calibration error on the NOVA machine. This also led to an error in the osmolality measurement, so this data point was also not considered, as will be

discussed below. Since the hollow fiber membrane cartridge was quite effective at removing lactate from the system, it is difficult to determine whether the glucose consumed by the Chinese hamster ovary cells was used primarily for cellular metabolism or the production of IgG_1 monoclonal antibody. Since the lactate concentration was significantly lower towards the later part of the experiment even though it was maintained at 2.5 g/L earlier during the perfusion segment, it may be assumed that at least a portion of the glucose consumed by the cells was directed towards monoclonal antibody production and less lactate was produced from cellular metabolism overall. This assumption makes intuitive sense because the CHO cells were undergoing significant shear stress created by the ATF system and these cells typically produce more antibodies when under stress.

The initial glutamine concentration in the HPCHO ATF experiment was nearly 6 mmol/L. The concentration of this metabolite decreased rapidly to less than 1.2 mmol/L by 72 hours. Perfusion of the ATF system was initiated at this time. Feeding of fresh medium and harvesting of spent medium from the reactor allowed the concentration of glutamine to increase to around 2.5 mmol/L. On average, the glutamine concentration remained around this level for the remainder of the experiment. There were some oscillations in the levels of this nutrient due to changes in perfusion rate and working volume, but the glutamine concentration never decreased below 1 mmol/L and never increased above 3 mmol/L. It is interesting to note that the amount of glutamine compared to the amount of glucose consumed was less in the ATF experiment than in the SCMR experiment. This may lend support to the assumption that the Chinese hamster ovary cells in the ATF experiment were utilizing more glucose for the production of monoclonal antibody than the HPCHO cells in the SCMR experiment. Further evidence of this assumption lies in the trend of ammonium ion concentration of the ATF experiment. At inoculation, the concentration of ammonium ion in the medium of the HPCHO culture was almost 1.3 mmol/L. The amount of this toxic waste product increased to about 5.2 mmol/L by 72 hours. Once perfusion of the ATF system was initiated at 72 hours, however, the ammonium ion concentration decreased to an average value of 3.3 mmol/L for the remainder of the experiment. Because high levels of ammonium ion may hinder the uptake of glucose by CHO cells, the fact that the ATF

system effectively decreased the levels of this waste product in the bioreactor probably allowed the HPCHO cells to take up more glucose for the purpose of producing IgG_1 monoclonal antibody.

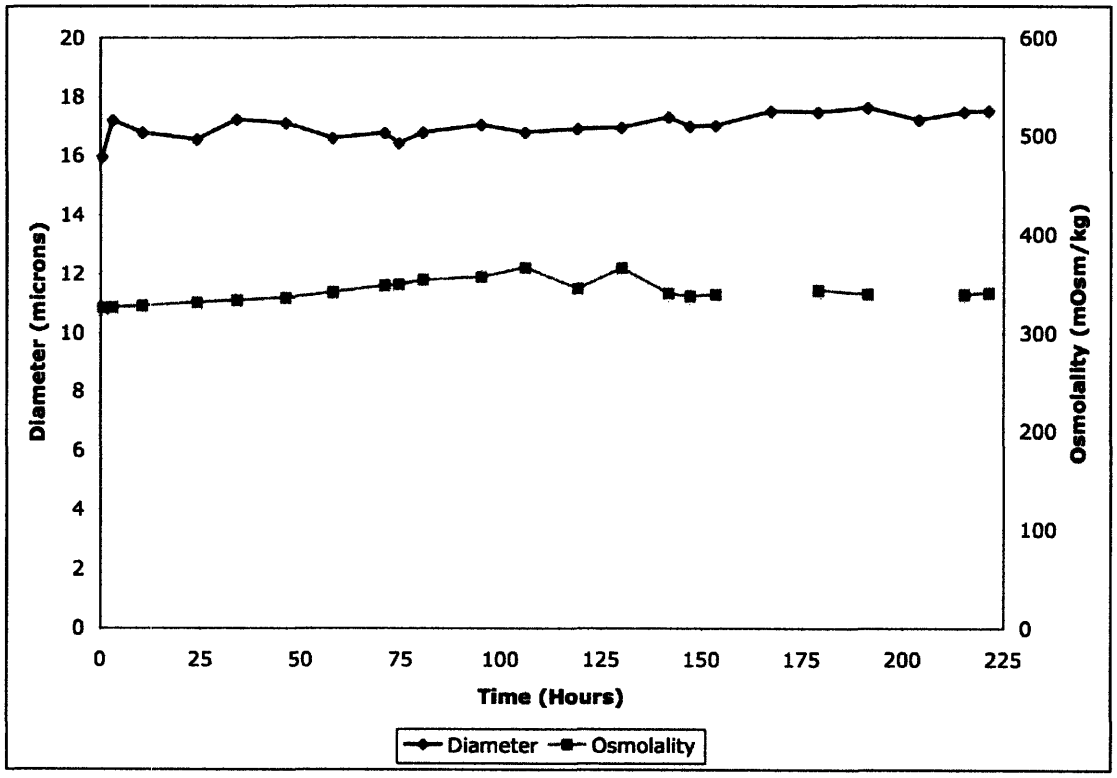

Figure 30 – Diameter and Osmolality Data for HPCHO ATF Run. The discontinuities in osmolality data at 168 and 204 hours are due to calibration errors with the NOVA machine.

The theory that cell diameter and culture osmolality trends could provide insight into the monoclonal antibody productivity of Chinese hamster ovary cells was discussed previously. The diameter and osmolality data for the HPCHO ATF experiment are shown in Figure 30. The graph shows little change in average cell diameter over the course of the ATF experiment. For most of the run, the average cell diameter ranged from around 16.5 microns to around 17.5 microns. The initial osmolality of the medium was calculated as around 325 mOsm/kg. The osmolality did not increase very much over the course of the experiment. In fact, the maximum osmolality for the culture was 365 mOsm/kg just after perfusion of the system was initiated. The osmolality determined by the NOVA machine at 168 and 204 hours were not considered due to errors in calibration of the lactate membrane. The osmolality value is calculated by the NOVA machine based on various metabolic concentrations, and if there is an error in these

concentrations, the resultant osmolality data is also in error. The osmolality of the HPCHO ATF culture over the course of this experiment was much lower than for any of the previous experiments with the Chinese hamster ovary cells. The osmolality trends show that the hollow fiber membrane was effective at removing excess salts from the bioreactor. Any stress felt by the cells during the ATF experiment was most likely due to shear rather than osmolality.

When qualitatively evaluating the monoclonal antibody production of the various HPCHO feeding strategies, it is necessary to consider three issues: the maximum viable cell concentration, the utilization of glucose, and the stress caused by increases in the osmolality of the medium. The maximum viable cell concentration of the HPCHO ATF experiment was quite similar to that of the SCMR experiment, and both of these perfusion experiments achieved a much higher cell concentration than either the batch or fed-batch experiments. The ATF experiment also produced more total cells than the SCMR experiment, but the viability of these cells was affected by the shear forces created by the ATF system. Study of the glucose and lactate levels in the CHO cultures over time showed that more glucose was utilized for monoclonal antibody production for both the SCMR and ATF perfusion experiments than in the batch experiment, but less glucose was used for this purpose in the perfusion experiments than in the first fed-batch experiment. It can be concluded that the individual cells in the perfusion experiments produced less monoclonal antibody than the individual cells in the first fed-batch experiment. This is further substantiated by the fact that the osmolality of the medium and the average cell diameter increased significantly in the fed-batch run but not in either of the two perfusion experiments. However, there were many more cells in the perfusion experiments than in the first fed-batch experiment, so total monoclonal antibody production was highest in the perfusion experiments. Closer comparison of the metabolite concentrations show that the ATF culture may have produced more monoclonal antibody than the SCMR culture, due to the shear stress created by the oscillating flow through the hollow fiber membrane cartridge. Again, it is important to note that the IgG1 monoclonal antibody produced by the HPCHO Chinese hamster ovary cells in either of the perfusion experiments may be more degraded than the product from experiments involving other feeding strategies. Differences in glycosylation of

monoclonal antibodies have been observed in cultures grown by different feeding strategies (33).

3.5 External spin filter system

Several perfusion experiments involving the external spin filter (ESF) system were performed with the HPCHO Chinese hamster ovary cell line as described above. The first goal of experimentation with the ESF equipment was to determine the optimal spin filter settings for perfusion of HPCHO cells. The rotational speed of the spin filter module was first tested. The first value investigated was 100 rpm. Since this was the rotational speed of the impeller of the bioreactor, the tests performed at 100 rpm were what could have been expected from an internal spin filter. The HPCHO cells were not well retained at this speed. Different rotational speeds of the spin filter were then tested, from 150 to 450 rpm. According to the manual for the spin filter apparatus, turbulent flow inside the apparatus began at rotational speeds of about 300 rpm. At this speed, greater retention of cells was observed, but at higher speeds, the viability of the culture decreased. An initial rotation speed of 300 rpm was therefore chosen for the full ESF perfusion run. The recirculation rate of the HPCHO culture was then tested. According to the manual for the spin filter apparatus, the recirculation rate must be kept at least at twice the speed of the perfusion rate to ensure proper recirculation of the culture. For low perfusion rates (1.5 vvd or lower), a recirculation rate of 100 rpm was found to be the most appropriate for the HPCHO culture. For higher perfusion rates (1.5 vvd or above), a recirculation rate of 200 rpm was found to be the most appropriate for the HPCHO culture. From information gathered from the previous perfusion experiments with HPCHO cells, minimal testing of perfusion rates was needed. The optimal set of feeding rates found for ESF perfusion culture of HPCHO Chinese hamster ovary cells in a 50-50 mixture of BD CHO Medium (BD Biosciences, MD) and Sigma-Aldrich Ex-Cell Animal Component Free CHO Medium (Sigma-Aldrich Corp., MO) is shown in Table 5. The perfusion rates for the ESF experiment do not exactly match the perfusion rates for the SCMR or ATF experiments. It is important to note that every attempt was made to significantly increase the perfusion rate at set stages, but due to aggregation of the

Chinese hamster ovary cells as well as fouling of the steel external spin filter module, the perfusion rates were often lower than what had been expected. Since the perfusion rates were consistently 1.5 vvd or lower, a recirculation rate of 100 rpm was utilized in the HPCHO ESF experiment.

Table 5 – Actual Set of Perfusion Rates and Spin Filter Rotation Speeds for ESF Culture of HPCHO Cells

Time	Perfusion Rate (Volume of Fresh Medium / Working volume / Day)	Spin Filter Rotation Speed
0 -- 71 hours	0 vvd	0 rpm
71 – 95 hours	0.9 vvd	300 rpm
95 – 104 hours	1.2 vvd	300 rpm
104 – 118 hours	1.3 vvd	300 rpm
118 – 165 hours	1.4 vvd	400 rpm

The most successful ESF experiment completed with the HPCHO Chinese hamster ovary cell line will be discussed. The three-liter bioreactor was inoculated with a 1.8-liter working volume of HPCHO culture with an initial density of around 1.5×10^5 cells/mL. Disturbances included the purging of the sample line of 3 mL and the sampling of 5 mL twice daily, as well as feeding of fresh medium and harvesting of spent medium after 71 hours. The autoclaved 3 liter bottles that contained the fresh medium to be fed and that collected the spent medium were replaced with a new set of autoclaved bottles after 140 hours. The total amount of medium supplied to the HPCHO ESF experiment was 7.1 liters.

The cell concentration and viability data for the HPCHO ESF experiment are shown in Figure 31. The ESF culture experienced a lag phase until around 30 hours. The lag phase was due to the adjustment of the CHO cells to the new environment of the stirred tank reactor. Because this period was similar in length to the lag phases in the batch, SCMR, and ATF experiments with the Chinese hamster ovary cells, it was concluded that the ESF equipment set-up had little to no initial effect on the growth and viability of the HPCHO cells. This was to be expected since the ESF system is primarily an external perfusion system. As will be discussed below, the ESF experimental design would have a major effect on the growth and viability of the HPCHO cells in the later

stages of the run and this effect would be related to the rotational speed of the spin filter module.

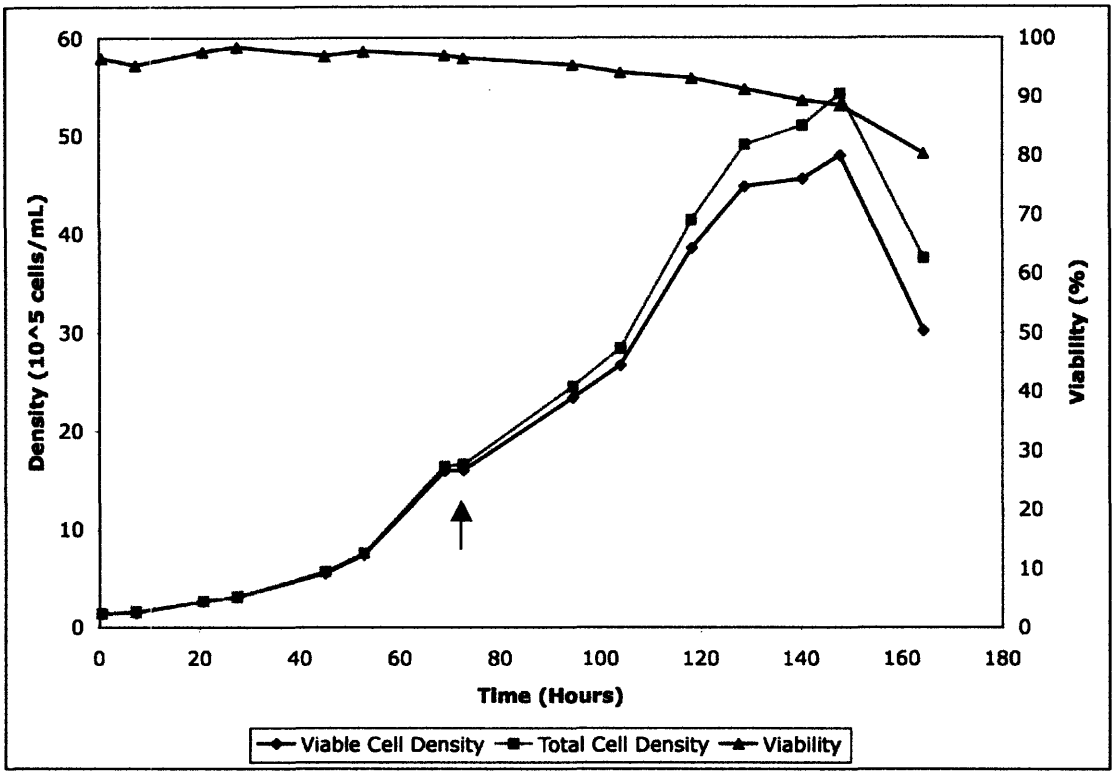

Figure 31 – Growth Data for HPCHO ESF Run. The black arrow indicates the start of perfusion.

The exponential growth phase of the HPCHO ESF experiment began around 30 hours and continued until about 130 hours. The average growth rate of the Chinese hamster ovary cells over the entire exponential phase was 0.026/hr. The exponential growth phase of the HPCHO ESF experiment, however, appeared to have two different regions. The first region began at 30 hours and continued until 69 hours. This period represented the growth of the cells possible during the batch phase of the experiment. The average growth rate during this time period was 0.037/hr. This value was higher than the batch growth rate of 0.026/hr, the first fed-batch growth rate of 0.029/hr, the growth rate of the first region of the exponential growth in the SCMR system of 0.031/hr, and the growth rate of the first region of the exponential growth in the ATF system of 0.032/hr, but significantly less than the growth rate of the second fed-batch experiment of 0.045/hr. This confirmed that the ESF experimental design had no initial effect on the growth and viability of the HPCHO Chinese hamster ovary cells. The maximum growth rate during the first exponential growth region of the HPCHO ESF experiment was

0.047/hr. The second region of exponential growth began around 69 hours and continued until 130 hours. The average growth rate during this time period was 0.015/hr. This represented the growth of the cells possible during the perfusion phase of the experiment. This growth rate was much lower than the batch and fed-batch growth rates, as well as the growth rate associated with the perfusion phase of the SCMR experiment of 0.021/hr. The growth rate associated with the perfusion phase of the ESF experiment was greater than that for the ATF experiment of 0.008/hr. Although feeding fresh medium and harvesting spent medium from the reactor with the ESF system allowed the HPCHO cells to continue growing at a reduced rate, this rate was not as impressive as the rate attained in the HPCHO SCMR experiment. The exponential growth phase in the ESF experiment was also much shorter than those of the other perfusion experiments. The ESF culture exhibited a stationary phase from 130 hours to 148 hours. After this time, a death phase was seen. These phases of the cell cycle were not clearly detected in the SCMR and ATF cultures.

The maximum viable cell concentration reached by the HPCHO ESF experiment was 48.01 x 10^5 cells/mL at 148 hours. This value was only slightly higher than the maximum viable cell concentrations reached by the HPCHO batch and fed-batch experiments and was much less than half the maximum viable cell concentrations reached by the HPCHO SCMR and ATF experiments. If the IgG_1 monoclonal antibody production of the HPCHO ESF system were qualitatively evaluated based on maximum viable cell concentration, it would be slightly higher than that of the batch and fed-batch systems but much less than that of the SCMR and ATF systems. The maximum total cell concentration achieved by the HPCHO ESF experiment was 54.26 x 10^5 cells/mL at 148 hours. Again, this value was not as impressive as the total cell concentrations reached in the other perfusion experiments.

During the ESF experiments with the Chinese hamster ovary cells, it was discovered that a large proportion of cells continued to pass through the steel filter module and appeared in the harvest bottle of the system. Although a small proportion of cells appeared in the harvest of the other perfusion systems, the proportion was small enough to be negligible and did not affect the total densities determined in the bioreactor. This phenomenon appeared to be related to the rotational speed of the external spin filter

module. The experiment began with an ESF rotational speed of 300 rpm, but so many cells were being harvested rather than replaced into the bioreactor, that the rotational speed was increased to 400 rpm. At this point, the viability of the cells suffered due to the increased shear forces created by the increased rotational speed. Table 6 shows the two rotational speeds with the respective density found in the bioreactor, density found in the harvest bottle, and viability of the cells in the bioreactor.

Table 6 – HPCHO ESF Rotational Speeds and Corresponding Cell Densities in the Reactor and Harvest Bottle, and Cell Viability in the Reactor

ESF Rotational Speed	Total Density of Cells in Reactor	Total Density of Cells in Harvest	Viability of Cells in Reactor
300 rpm	16.60×10^5 cells/mL	1.55×10^5 cells/mL	96.6 %
300 rpm	28.38×10^5 cells/mL	7.80×10^5 cells/mL	94.1 %
400 rpm	54.26×10^5 cells/mL	0.74×10^5 cells/mL	88.5 %

As Figure 31 and Table 6 clearly illustrate, the external spin filter affected the viability of the Chinese hamster ovary cells. The viability of the HPCHO culture immediately after inoculation was quite high and remained above 95% for 95 hours. Feeding of fresh medium and harvesting of spent medium was initiated after 71 hours. Harvesting of spent medium involved pumping cells and medium from the reactor, and then forcing this mixture through the external spin filter apparatus. After about 20 hours of perfusion operation, the viability of the culture began to decrease. By 140 hours, the viability of the HPCHO culture had decreased below 90%. The viability of the cells may have been related to the rotational speed of the external spin filter. When the rotational speed of the spin filter was increased from 300 rpm to 400 rpm in an attempt to prevent more cells from passing through the 20 μm filter module, the viability of the culture decreased more rapidly. By the end of the experiment after 165 hours, the viability of the HPCHO culture had decreased to 80%. The decreased viability of the cells may have created or worsened the cell aggregation problem. This aggregation of the HPCHO cells eventually led to clogging of the tubing containing the culture as well as the base of the external spin filter apparatus.

Major metabolite concentrations of the HPCHO ESF culture are illustrated in Figure 32. At inoculation, the glucose concentration was 5.25 g/L. The glucose

concentration in the ESF culture decreased steadily until it reached 3.5 g/L after 69 hours. The initiation of perfusion at 71 hours increased the glucose concentration to 3.8 g/L. After this point, the glucose was consumed more rapidly by the cells and decreased to 0.7 g/L by 165 hours. There was no lactate present in the ESF culture at inoculation. After 20 hours, the lactate concentration in the culture increased steadily until it reached 1.65 g/L. Although this amount of lactate was slightly less than the amount of glucose consumed by the HPCHO cells, it can be concluded that most of the glucose during the batch phase of the experiment was used for cell metabolism and growth rather than production of monoclonal antibody. When perfusion was initiated after 71 hours, the lactate concentration remained constant, showing that the ESF system was at first effective in removing excess lactate from the culture. After 120 hours, the lactate concentration began increasing and it reached 3.5 g/L by the end of the experiment. It is difficult to determine if the glucose consumed during the perfusion phase was utilized in cell metabolism or antibody production. If the glucose in the medium was replaced at the same rate as the lactate was removed, however, it is important to note that the glucose concentration decreased faster than the lactate concentration increased. As previously discussed, the HPCHO cells were under considerable stress caused by the shear forces of the external spin filter module. Therefore, it is possible that much of the glucose consumed during the perfusion phase of the ESF experiment was utilized for the production of IgG_1 monoclonal antibody.

At inoculation the glutamine concentration of the HPCHO ESF experiment was over 5.1 mmol/L. During the batch phase of the experiment, the glucose concentration decreased steadily until it was 2.4 mmol/L after 69 hours. Feeding of fresh medium and harvesting of spent medium was initiated at 71 hours. After this time, the glutamine concentration initially increased to 2.91 mmol/L. For the perfusion phase of the experiment, the glutamine concentration decreased slowly, but ended at a final value of 2.5 mmol/L. The fact that the glutamine concentration decreased so slowly during this period verifies the theory that glucose was preferentially consumed by the HPCHO cells for the purpose of producing IgG_1 monoclonal antibody. The concentration of ammonium ion in the ESF culture at inoculation was about 1.4 mmol/L. During the batch phase of the experiment, the ammonium ion concentration increased quickly to

about 4.2 mmol/L at 69 hours. After perfusion was initiated, the ammonium ion concentration initially dropped to 3.6 mmol/L. This showed that the external spin filter module was effective at removing the toxic waste product from the system. The concentration of ammonium ion was maintained at this value and then decreased to a final value of 3 mmol/L. The fact that the ammonium ion concentration was maintained at such a low value in the ESF culture helped the HPCHO cells take up glucose for cell metabolism as well as the production of monoclonal antibody.

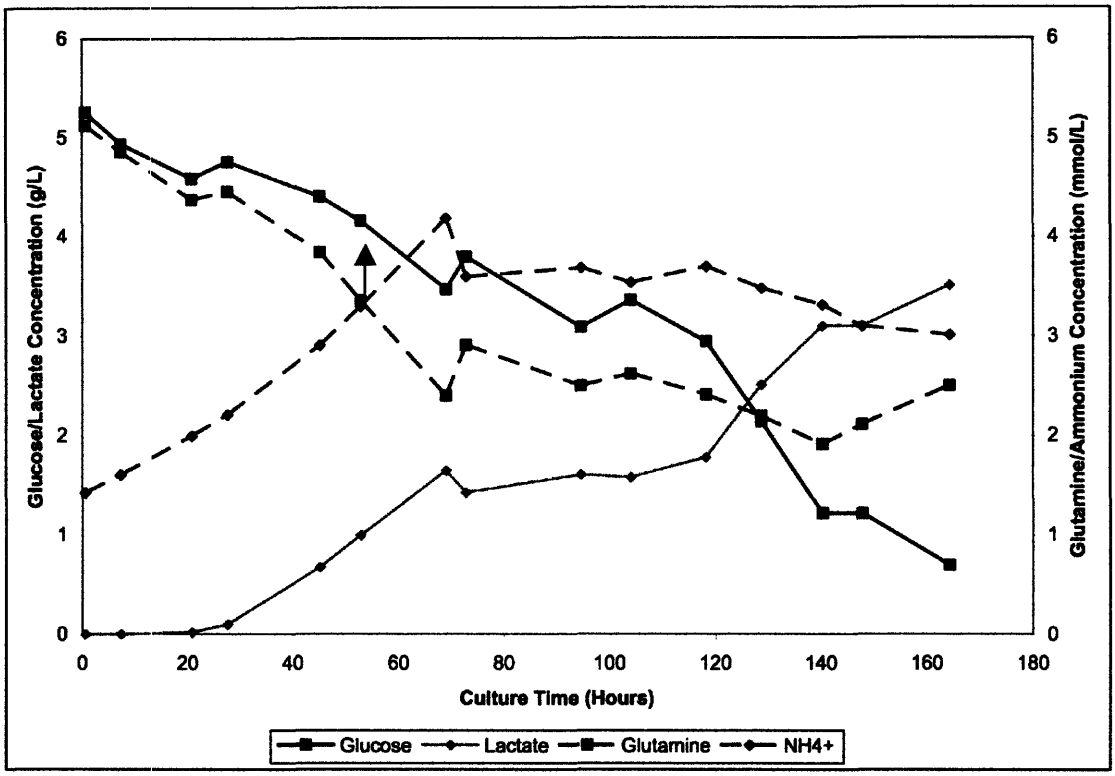

Figure 32 – Metabolic Data for HPCHO ESF Run. The black arrow indicates the start of perfusion.

The average cell diameter and osmolality of the culture medium over time for the ESF HPCHO experiment was quite similar to that of the batch culture. The average cell diameter remained between 16.5 and 17.5 microns for the entirety of the culture. The osmolality of the culture medium began at around 325 mOsm/kg. By 150 hours, the osmolality had increased to about 360 mOsm/kg. After the rotational speed of the external spin filter module was increased from 300 rpm to 400 rpm, the osmolality quickly increased to 415 mOsm/kg. However, this increase in osmolality was most likely not related to increased monoclonal antibody production, but rather the shear forces generated by the spin filter module that also decreased the viability of the HPCHO cells.

The production of the ESF experiment involving the Chinese hamster ovary cells can be compared to that of the first fed-batch experiment. Although the maximum viable cell concentration of the ESF culture was not much higher than that of the batch culture, the HPCHO cells were under considerable stress and probably produced more monoclonal antibody per cell. However, the maximum viable cell concentration of the ESF culture was much lower than that of the other perfusion systems. Even if the cells produced more monoclonal antibody per cell, the vastly smaller amount of cells would lead to a total amount of monoclonal antibody produced that was less than that produced by the other perfusion systems.

4 Experiments with IB4 Hybridoma Cells

4.1 Batch system

A batch experiment was performed with the IB4 hybridoma cell line in the manner described above. The three-liter bioreactor was inoculated with a 1.8-liter working volume of cell culture at a density of around 1.5 x 10^5 cells/mL. The only disturbance to the culture was the purging of the sample line of 3 mL and the sampling of 5 mL twice daily. The total amount of medium supplied to the IB4 batch experiment was 1.8 liters.

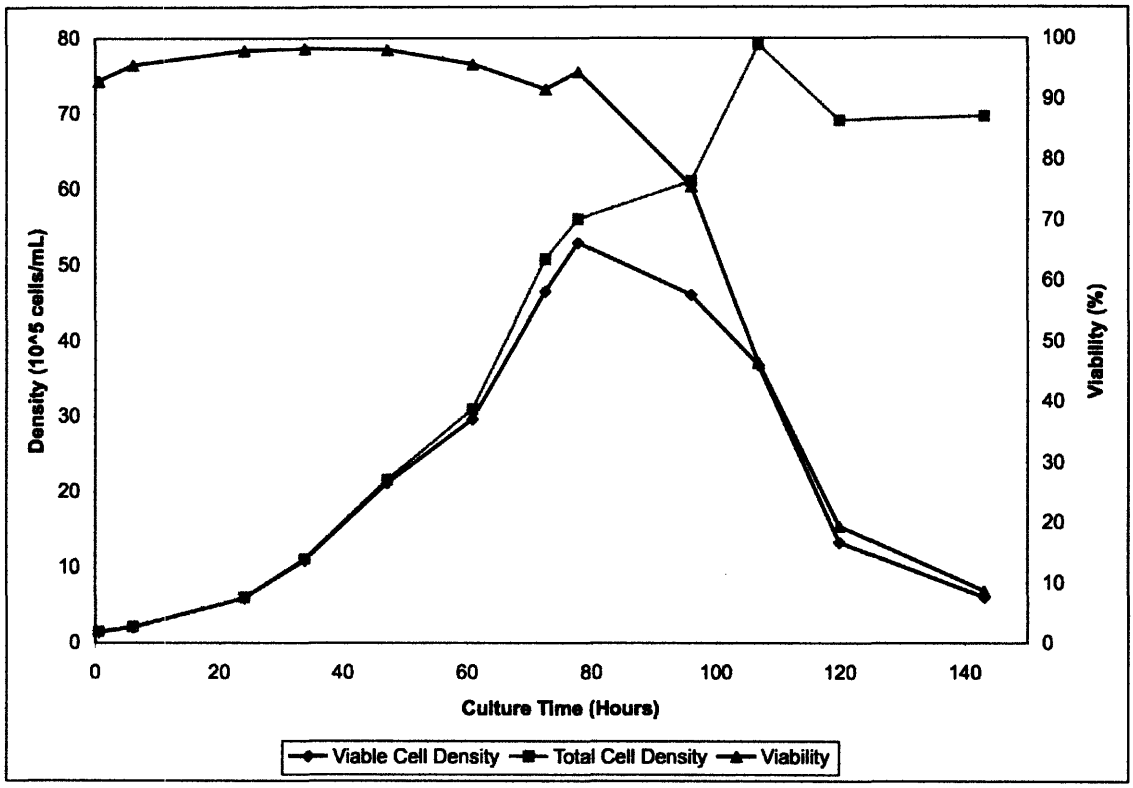

Figure 33 – Growth Data for IB4 Batch Run

The cell concentration and viability data for the batch experiment are shown in Figure 33. The culture experienced a lag phase until around 25 hours. During this time, the hybridoma cells were adjusting to the change in environment resulting from transitioning from the spinner flask to the stirred tank reactor. Exponential growth of the culture began around 25 hours and continued until around 80 hours. The average growth

rate during this period was 0.04/hr. The maximum growth rate during this period was 0.063/hr and occurred at the beginning of the exponential growth phase around 35 hours. The culture experienced a stationary phase from around 80 hours to about 100 hours. A death phase began around 100 hours and continued until about 145 hours, when the viability of the cells dropped below 10% and the experiment was ended.

The maximum viable cell concentration reached by the batch hybridoma culture was 52.92 x 10^5 cells/mL at about 80 hours. The maximum total cell concentration achieved was 79.15 x 10^5 cells/mL at 105 hours. The viability of the culture began slightly lower than normal, at 93% rather than above 95%, but was very high throughout the lag, exponential growth, and stationary phases. The viability of the cells remained above 90% until almost 100 hours. The IB4 hybridoma cells began dying after this time point.

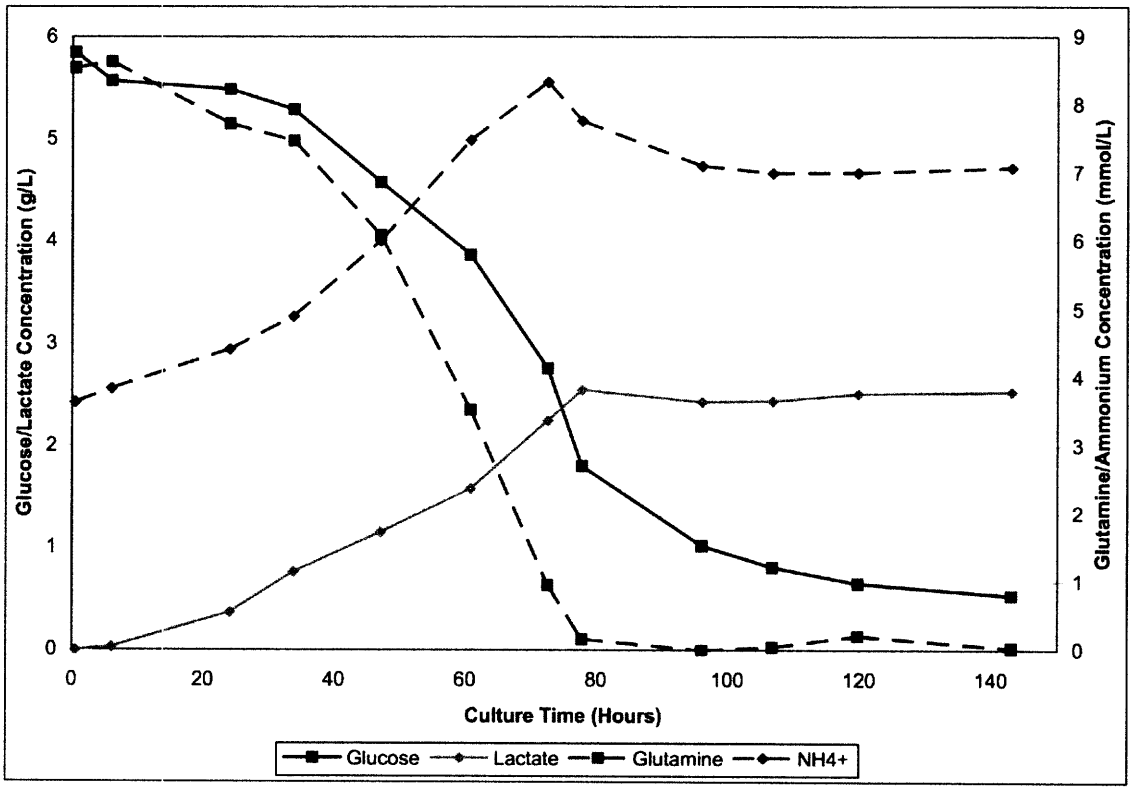

Figure 34 – Metabolic Data for IB4 Batch Run

The major metabolite concentrations of the batch hybridoma culture are shown in Figure 34. The glucose concentration began at around 6 g/L, and was reduced to below 1 g/L by 100 hours. The lactate concentration began at 0 g/L and increased to around 2.5

g/L by 80 hours. After this point, the lactate concentration maintained this concentration until the end of the experiment at 145 hours. The increases in lactate concentration corresponded to the decreases in glucose concentration. The lactate concentration stopped increasing as soon as the glucose concentration fell to significantly low levels. The glutamine concentration began at around 8.5 mmol/L and was reduced to below 0.2 mmol/L by 75 hours. The concentration of ammonium ion began at around 3.5 mmol/L and increased until it reached nearly 8.5 mmol/L at 75 hours. There was a slight decrease in ammonium ion concentration from 75 to 100 hours, possibly due to the fact that the glutamine concentration fell to significantly low levels at this point. The ammonium ion concentration then reached a plateau at about 7 mmol/L after 100 hours, probably due to cell death. It appears that the culture utilized glutamine at a faster rate than it used glucose, however, this is incorrect because there was less glutamine than glucose available to the culture at inoculation. It is apparent from these trends in metabolite concentrations that feeding in fed-batch or perfusion experiments should begin before the 80 hour time point. At this time, the glutamine concentrations became significantly decreased and the consumption of glucose by the cell culture was at its highest rate.

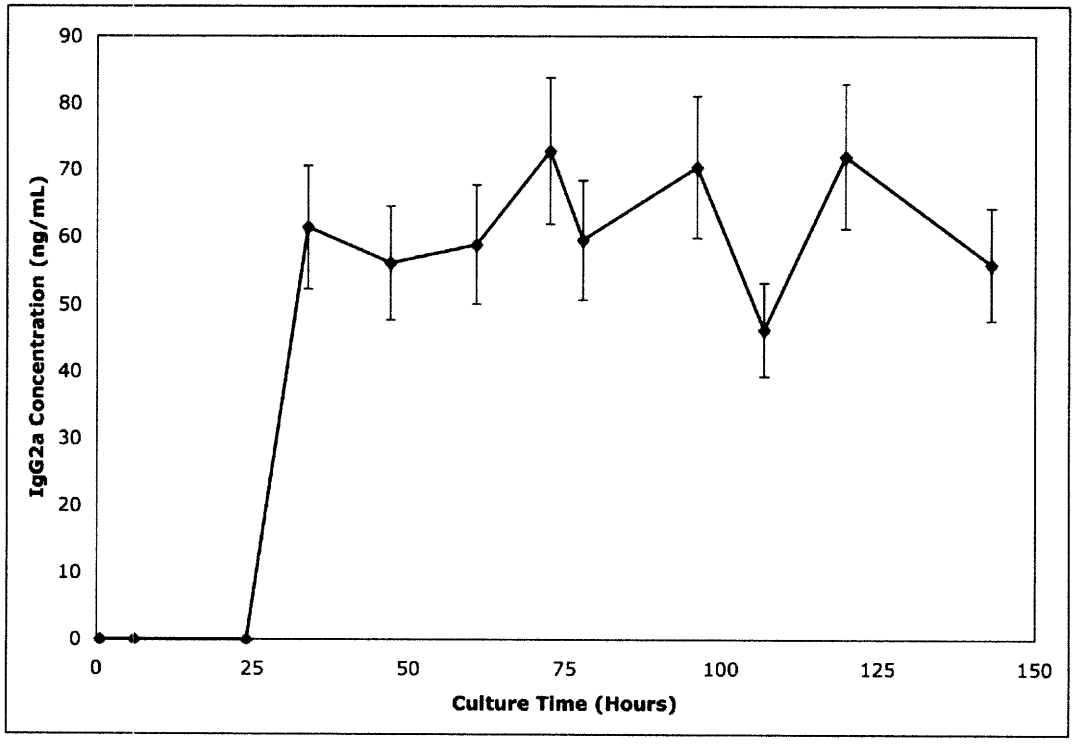

Figure 35 – Monoclonal Antibody Concentration Data for IB4 Batch Run. The error bars correspond to one standard deviation.

The IgG_{2a} monoclonal antibody concentration of the IB4 batch experiment was determined periodically. The capillary electrophoresis data illustrating the concentration of IgG_{2a} monoclonal antibody over time in the hybridoma batch culture is shown in Figure 35. During the lag phase of the culture, there were no detectable levels of monoclonal antibody in the reactor. Once the hybridoma cells entered the exponential growth phase, they also began producing small amounts of monoclonal antibody. At 34 hours, there was an antibody concentration of over 60 ng/mL. By 72 hours, or a few hours before the batch culture reached its maximum viable cell density, the antibody concentration had increased to over 70 ng/mL. After this point, the antibody concentration remained between 60 and 70 ng/mL for the remainder of the culture. There was some variability in the samples, but the qualitative trend should be trusted because the error bars overlap.

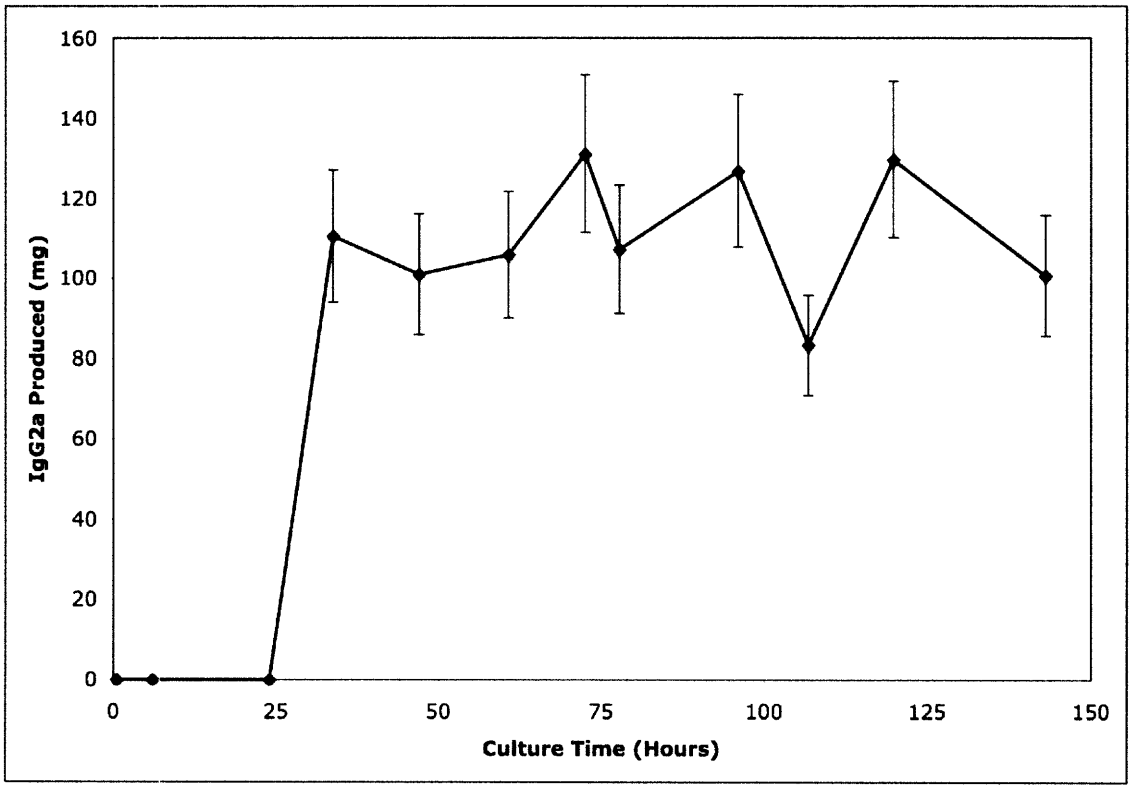

Figure 36 – Total Amount of Monoclonal Antibody Produced vs. Time for IB4 Batch Run. The error bars correspond to one standard deviation.

The total amount of monoclonal antibody produced over time by the IB4 hybridoma batch run is shown in Figure 36. The qualitative trend in this graph is quite similar to that of Figure 35. No antibody was produced until after the lag phase. Over

110 mg of antibody was produced by 35 hours. A maximum amount of around 130 mg of antibody was produced by 72 hours. After this point, the total amount of antibody in the 1.8 L working volume of the reactor remained between 110 and 130 mg of total IgG_{2a}. From these data, it is apparent that the IB4 hybridoma cells produce the most monoclonal antibody during the exponential growth phase of the batch culture. The monoclonal antibody production, growth, viability, and metabolite concentration of the IB4 batch culture will be used as a baseline to compare results gathered from the other IB4 experiments with differing feeding strategies.

4.2 Fed-batch system

Several fed-batch experiments were performed with the IB4 hybridoma cell line as described above. Initial fed-batch experiments were performed with various starting volumes ranging from 1 L to 1.65 L of BD Cell mAb Medium Quantum Yield (BD Biosciences, MD). The medium was supplemented as previously described. It was found that 1.65 L of medium allowed the cells to grow in a matter most similar to that in the early stages of the batch experiment. This was due to the need for the volume of the culture to be great enough to completely immerse the two sets of impeller blades on the drive shaft of the reactor. This volume was similar to the desired volume determined by the initial fed-batch experiments with the HPCHO Chinese hamster ovary cell line. The initial fed-batch experiments with the IB4 cells also showed that feeding of the culture should proceed after about 72 hours of growth and could be supplied in around 150 mL bulk doses via a sterile syringe. The necessary timing of the bulk doses was similar to that found in the fed-batch experiments with HPCHO cells, but the volume of the bulk doses was nearly 50% larger in the fed-batch experiments with the IB4 hybridoma cell line. In addition to varying the initial volume and the volumes of the bulk feeding doses, the supplementation of the feed medium was varied. Experiments were performed with feed medium that were supplemented as above but with no additional L-glutamine, feed medium that was supplemented as above but also with concentrated essential amino acid solution, and other variants. In all cases, the feed medium was concentrated via Rota-vapor apparatus from 1 L to 300 mL. It was found that the optimal feed medium was a

concentrated form of the medium used to inoculate the initial volume of cell culture (the BD Cell mAb Medium Quantum Yield supplemented as described in the experimental design section dealing with cell lines and medium).

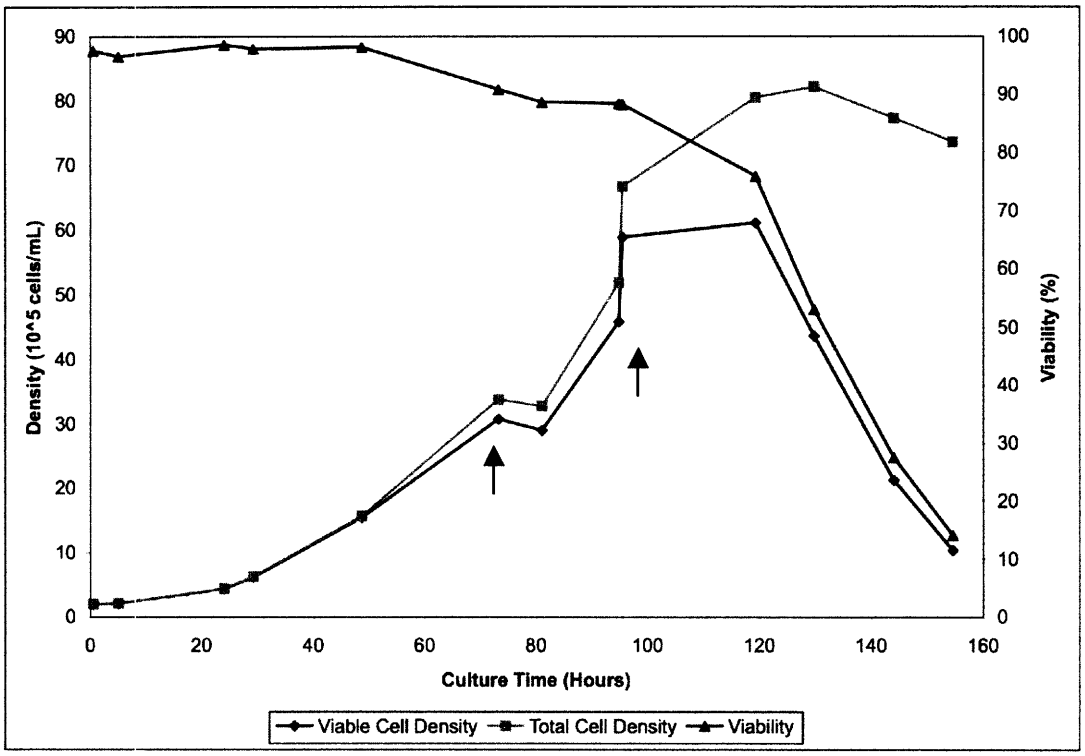

Figure 37 – Growth Data for IB4 Fed-Batch Run. The black arrows indicate feeding doses.

The most successful fed-batch experiment with the IB4 hybridoma cell line will be discussed. The three-liter bioreactor was inoculated with a 1.65-liter working volume of IB4 culture with an initial density of around 2.0 x 10^5 cells/mL. Disturbances included the purging of the sample line of 3 mL and the sampling of 5 mL twice daily, as well as two 150 mL bulk doses of feed medium delivered via sterile syringe at around 74 and 95 hours. When concentrated medium is considered, the total amount of medium supplied to the IB4 fed-batch system was 2.65 liters.

The cell concentration and viability data for the IB4 fed-batch experiment are shown in Figure 37. The culture experienced a lag phase until around 25 hours. During this time, the hybridoma cells were adjusting to the change in environment resulting from transitioning from the spinner flask to the stirred tank reactor. The lag phase in the fed-batch experiment was the same length as the lag phase in the batch experiment. Even though the initial volume of the fed-batch experiment was lower than in the batch

experiment (leading to a potential harsher environment for the cells), the inoculation density of the fed-batch culture was slightly higher than for the batch culture (2.0×10^5 cells/mL rather than 1.5×10^5 cells/mL). A graph showing the cell concentration data for both the batch and fed-batch hybridoma experiments is shown in Figure 38, so that the two experiments may be compared visually. The fed-batch data series have solid lines and the batch data series have dashed lines.

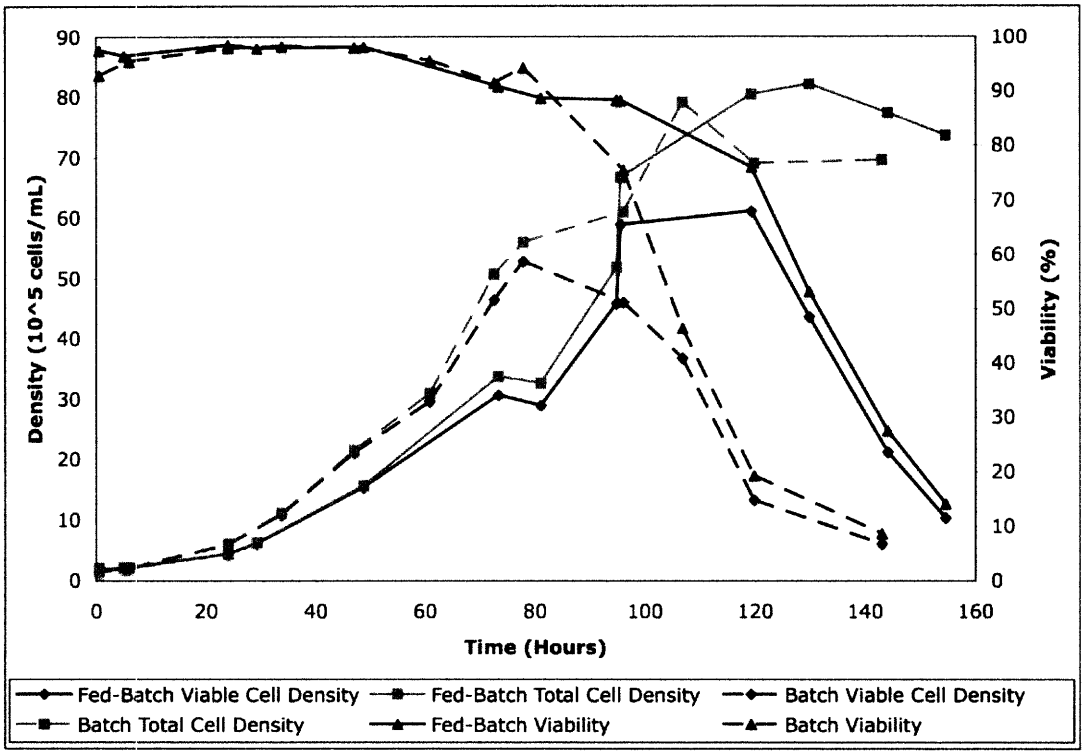

Figure 38 – Comparison of Growth Data for IB4 Batch Run and IB4 Fed-Batch Run

The exponential growth of the fed-batch culture began around 25 hours and continued until around 96 hours. The average growth rate during this period was 0.034/hr. The maximum growth rate during this period was 0.064/hr and occurred towards the end of the exponential growth phase around 90 hours. This value is suspiciously high, however. Although the density data at the 73-hour time point and the 96 hour time point appear to be correct, the density data in between these values are quite low. An alternate Cedex machine was used for these data points and may be the source of the error in the measurements. For this reason, only the average growth rate for the fed-batch experiment will be considered. The average growth rate of the fed-batch culture was slightly lower than that for the batch experiment (0.034/hr rather than

0.04/hr). The exponential growth phase was longer in the fed-batch experiment, however. The fed-batch culture experienced a stationary phase from around 96 hours to about 120 hours. Finally, the hybridoma fed-batch experiment experienced a death phase that began around 120 hours and continued until about 155 hours, when the viability of the cells dropped below 15% and the experiment was ended.

The maximum viable cell concentration reached by the fed-batch IB4 hybridoma culture was 61.15 x 10^5 cells/mL at 120 hours. The maximum total cell concentration achieved was 82.19 x 10^5 cells/mL at 130 hours. Both of these values are higher than what was achieved in the batch culture (a maximum viable cell concentration of 52.92 x 10^5 cells/mL at 80 hours and a maximum total cell concentration of 79.15 x 10^5 cells/mL at 105 hours) and this fact is well illustrated in Figure 38. The viability of the fed-batch culture was high throughout the lag and exponential growth phases, but decreased slightly during the stationary phase and plummeted during the death phase. The viability of the cells remained above 85% until 95 hours and remained above 75% until 120 hours. As Figure 38 illustrates, the viability of the fed-batch culture was similar to that of the batch culture. The viability of the fed-batch cells was maintained at a high value for a longer period of time than in the batch culture because the exponential growth phase of the fed-batch experiment was longer. The cells in the fed-batch culture began dying around 120 hours.

The concentrations of the major metabolites of the fed-batch IB4 hybridoma culture are shown in Figure 39. At the start of the culture, the glucose concentration was nearly 6 g/L. The concentration of glucose decreased slowly to about 4 g/L at 74 hours. The first bulk feed increased the glucose concentration to almost 4.5 g/L. From 80 hours to 120 hours, the glucose was consumed at a faster rate until it decreased below 1 g/L. The second bulk feed at 95 hours did not cause an increase in the observed glucose concentration of the medium several hours later, but probably did initially provide more glucose for uptake by the cells. The glucose concentration was reduced to 0 g/L by 145 hours. The trends in lactate concentration for the IB4 fed-batch experiment were interesting. The lactate concentration began at 0 g/L and increased to nearly 4.5 g/L by 130 hours. The increases in lactate concentration corresponded qualitatively to the decreases in glucose concentration. The lactate concentration stopped increasing at

around 130 hours as soon as the glucose concentration fell to significantly low levels. It is important to note that the amount of glucose taken up by the IB4 hybridoma cells was slightly greater than the amount of lactate produced by the cells. The maximum levels of the two metabolites differed by more than 1 g/L. This is evidence that the cells continued to take up glucose and used the nutrient to produce antibody rather than to undergo normal metabolism.

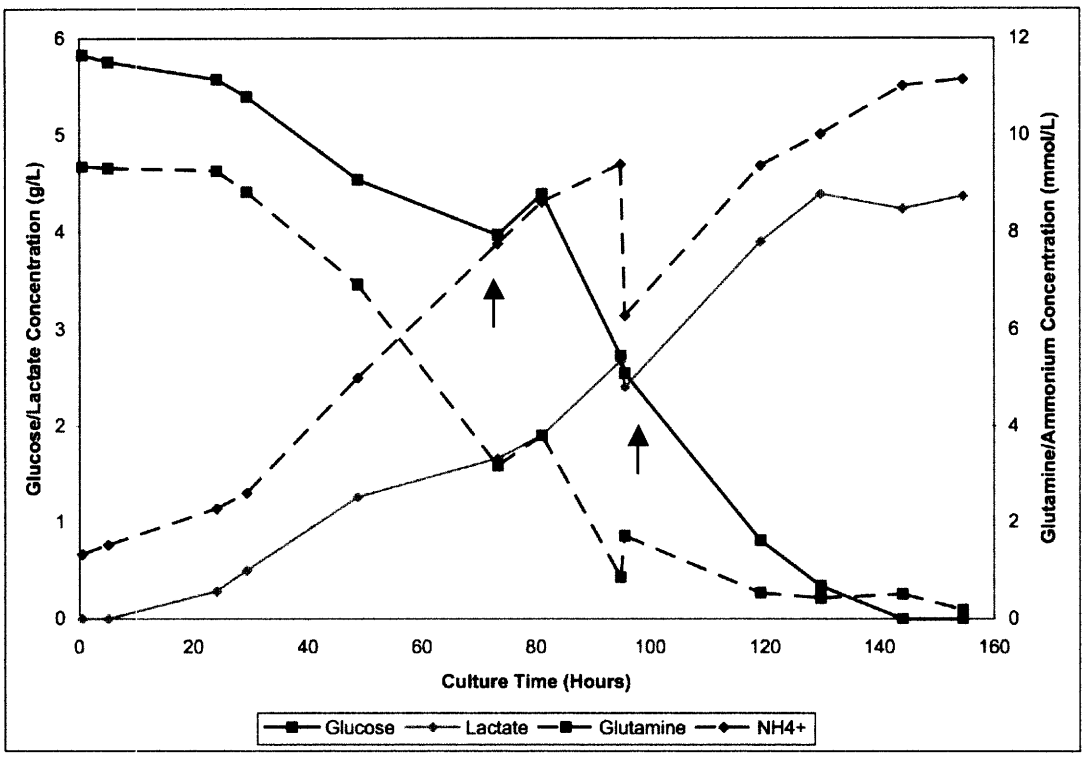

Figure 39 -- Metabolic Data for IB4 Fed-Batch Run. The black arrows indicate feeding doses.

The glutamine concentration of the IB4 hybridoma fed-batch culture began at around 9.5 mmol/L and was reduced to below 0.5 mmol/L by 120 hours. During the lag phase of the culture, from inoculation to 25 hours, there was little change in glutamine concentration. After 25 hours, the concentration of glutamine decreased at a relatively constant rate until the first bulk feeding at 74 hours. The bulk feeding increased the glutamine concentration from 3.2 mmol/L to 3.8 mmol/L. The glutamine concentration continued to decrease steadily until the second bulk feeding at 95 hours. The second feed increased the glutamine concentration from 0.9 mmol/L to 1.7 mmol/L. After the second feeding, the hybridoma cells consumed glutamine at a much slower rate. This time period was also when the cells were consuming glucose at a very high rate and the

stationary phase of the culture. The hybridoma cells were probably consuming glucose at this point to primarily produce monoclonal antibody rather than to continue growing and dividing. The concentration of ammonium ion began at around 1.5 mmol/L and increased until it reached over 11 mmol/L by 145 hours. The ammonium ion concentration increased steadily until the second bulk feeding at 95 hours. After this point, there was a significant drop in the concentration of the ion from 9.4 mmol/L to 6.8 mmol/L. This may be due to an error in the measurement with the NOVA machine, or perhaps due to the slight increase in culture volume created by the bulk feeding. If this odd decrease in ammonium ion concentration is ignored, it is apparent that after the second bulk feeding, the creation of ammonium ion decreased. The formation of ammonium ion after the second feeding was primarily due to cell death since the consumption of glutamine decreased significantly at that point.

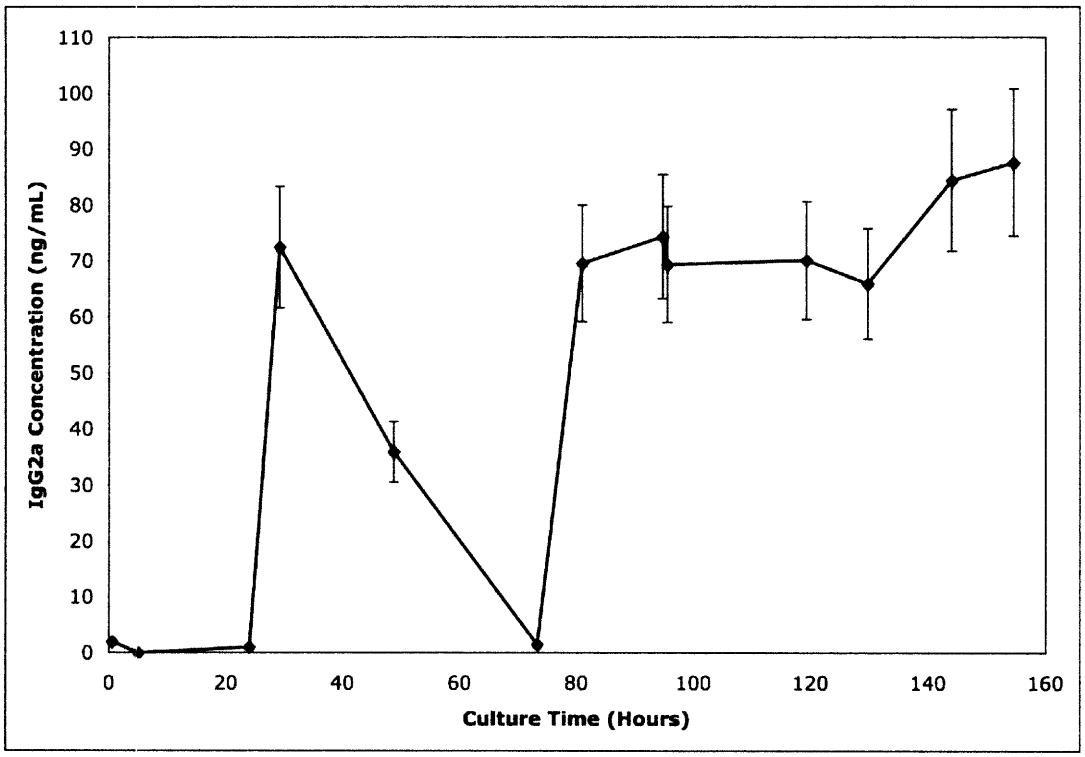

Figure 40 – Monoclonal Antibody Concentration Data for IB4 Fed-Batch Run. The error bars correspond to one standard deviation.

The IgG_{2a} monoclonal antibody concentration in the IB4 fed-batch culture was determined periodically. The capillary electrophoresis data illustrating the production of IgG_{2a} monoclonal antibody by the hybridoma fed-batch culture over time is shown in

Figure 40. There were no detectable levels of antibody while the hybridoma cells were in the lag phase. As the cells entered the exponential growth phase, they began producing measurable amounts of monoclonal antibody. At 30 hours, the concentration of IgG_{2a} in the reactor was over 70 ng/mL. After this point, the concentration of antibody apparently reduced to 0 ng/mL by 74 hours. Although the antibody could have degraded due to the temperature or other reactor conditions, it is likely that there was an error in measuring this sample rather than degradation of the antibody previously present in the reactor. When the fed-batch reaction was first fed after 74 hours, the concentration of monoclonal antibody began increasing from around 70 ng/mL at 80 hours to 75 ng/mL at 95 hours. After the fed-batch reaction was fed for the second time after 95 hours, the concentration of monoclonal antibody initially decreased slightly due to the increased working volume. At 96 hours, the concentration of IgG2a in the reactor was around 70 ng/mL. After this point, the antibody concentration gradually increased to over 87 ng/mL by the end of the culture. On average, the monoclonal antibody concentration in the fed-batch run was over 10 ng/mL greater than that of the batch experiment.

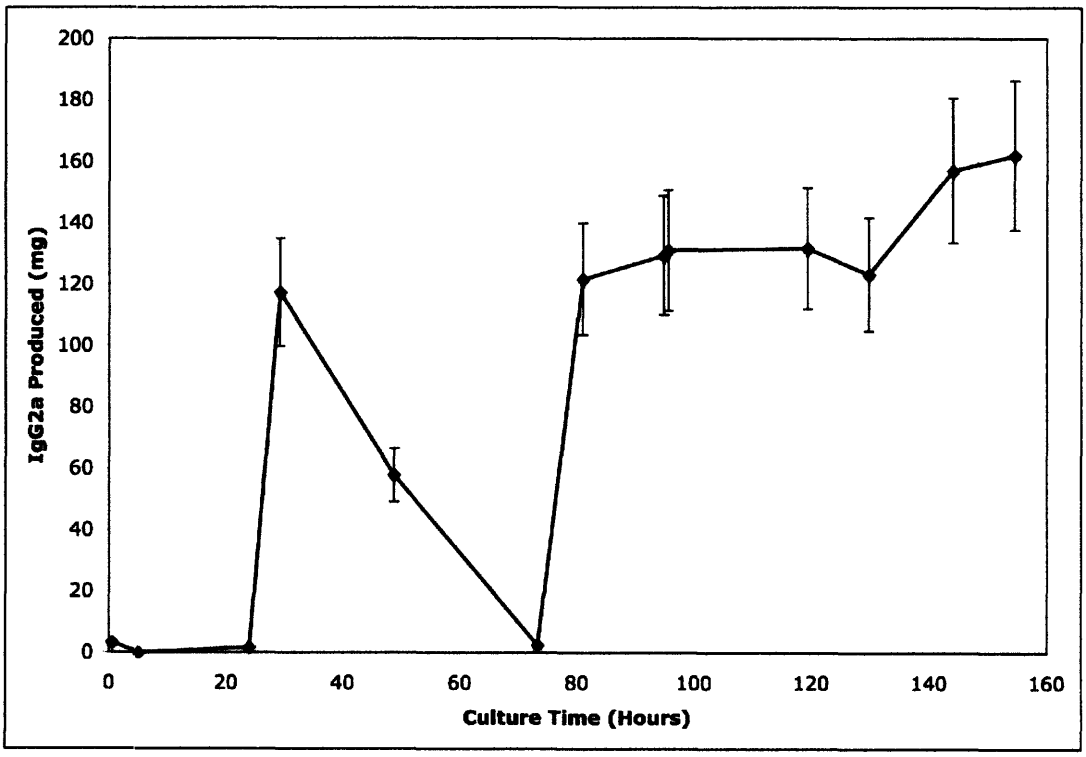

Figure 41 – Total Amount of Monoclonal Antibody Produced vs. Time for IB4 Fed-Batch Run. The error bars correspond to one standard deviation.

The total amount of monoclonal antibody produced by the IB4 fed-batch culture over time is shown in Figure 41. The qualitative trend illustrated by Figure 40 is mirrored in Figure 41. Initially, there was very little IgG2a produced by the reaction. Although there was some monoclonal antibody produced at the start of the exponential growth phase, the antibody production of the system truly started after the first bulk feed. By 80 hours, 120 mg of monoclonal antibody had been produced by the culture. The amount of antibody produced by the culture steadily increased until over 160 mg of IgG_{2a} had been produced by the end of the culture. The total amount of monoclonal antibody produced in the IB4 hybridoma fed-batch culture was 30 mg higher than the total amount produced in the batch experiment. The data show that feeding concentrated medium to the IB4 cells in two bulk doses during the exponential growth phase enhanced the total IgG_{2a} production of the system.

4.3 Ceramic membrane system

Several perfusion experiments involving the stirred ceramic membrane reactor (SCMR) system were performed with the IB4 hybridoma cell line as described above. A control experiment was first performed with the IB4 hybridoma cell line to determine the extent that the ceramic membrane module affected the culture environment of the bioreactor. In this experiment, the stirred ceramic membrane module was placed inside the three-liter bioreactor, but no feeding and harvesting of medium was initiated. The three-liter bioreactor was inoculated with a 1.8-liter working volume of IB4 culture with an initial density of around 1.5×10^5 cells/mL. The only disturbances to the hybridoma culture included the purging of the sample line of 3 mL and the sampling of 5 mL twice daily. The total amount of medium supplied to the IB4 control batch experiment was 1.8 liters.

Cell concentration and viability data for this control SCMR experiment with the IB4 hybridoma cell line are shown in Figure 42. The graph shows only one major difference between the IB4 batch experiment and this experiment that included the ceramic membrane module inside the bioreactor. The lag phase of the batch experiment containing the filter module was the same length as in the batch experiment with no filter

module (about 25 hours), but it was much more pronounced. Cell growth was quite limited until the exponential growth phase began at 25 hours. Consequently, the exponential growth phase lasted about 20 hours longer in the batch experiment containing the filter module, ending at around 95 hours instead of 75 hours as in the batch experiment. Much like the experiment with the HPCHO cells, the IB4 cells in the batch containing the ceramic membrane module died much more quickly than the IB4 cells in the batch without the filter module. Although the lag and death phases differed between the two experiments, there was no significant difference between the maximum viable cell concentrations and the maximum total cell concentrations in the two experiments. There were also no striking differences in the metabolite concentrations of the two experiments. It was concluded that the inclusion of the ceramic membrane module in the bioreactor had little to no adverse effect on the growth and viability of the IB4 hybridoma cell culture.

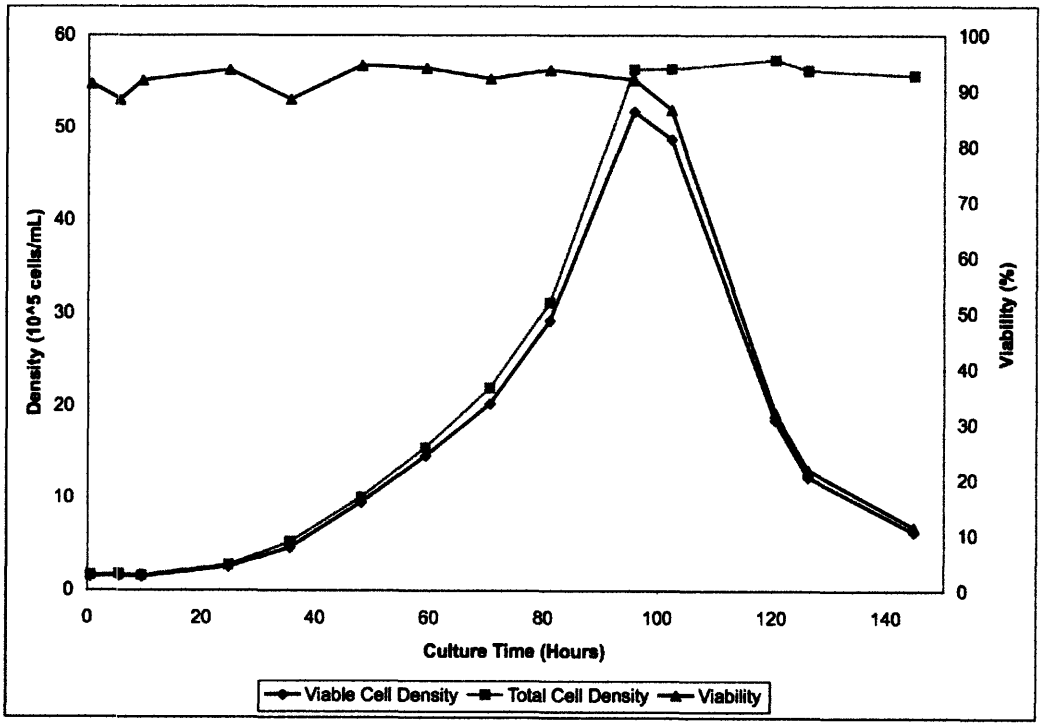

Figure 42 – Growth Data for IB4 SCMR Control Batch Run

The concentration of IgG_{2a} monoclonal antibody in the IB4 hybridoma batch culture containing the ceramic membrane module was determined periodically. The capillary electrophoresis data illustrating the antibody concentration in the control batch culture over time is shown in Figure 43. During the lag phase and the initial stages of the

exponential growth phase, there was a small level of about 30 – 60 ng/mL of monoclonal antibody in the reactor. After 50 hours, the concentration of IgG_{2a} increased significantly to 120 ng/mL at 60 hours. After this time, the concentration of antibody decreased to about 60 ng/mL. This decrease was similar to that seen in the fed-batch experiment and may be due to degradation of the antibody or error in the capillary electrophoresis measurement. Towards the end of the exponential growth phase, the concentration of antibody increases to 145 ng/mL at 120 hours. After this point, the product concentration decreases to over 85 ng/mL by the end of the experiment. This may be due to degradation of the antibody or error in the capillary electrophoresis measurement. A comparison of the maximum IgG_{2a} concentrations for the three hybridoma cultures discussed to this point shows that the control batch culture achieved a concentration twice as high as that achieved by the batch experiment and significantly higher than the maximum product concentration of 87 ng/mL achieved by the fed-batch experiment. The average antibody concentration towards the end of the control batch run was around 110 ng/mL, also higher than the average product concentrations at the ends of the batch and fed-batch cultures.

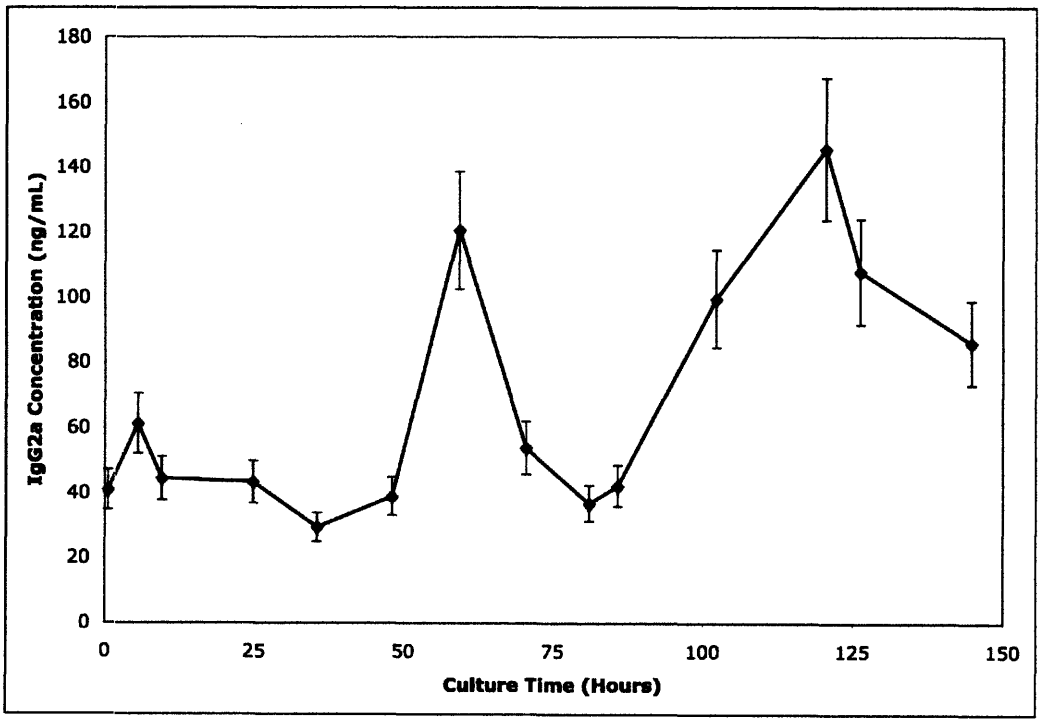

Figure 43 – Monoclonal Antibody Concentration Data for IB4 Control Batch Run. The error bars correspond to one standard deviation.

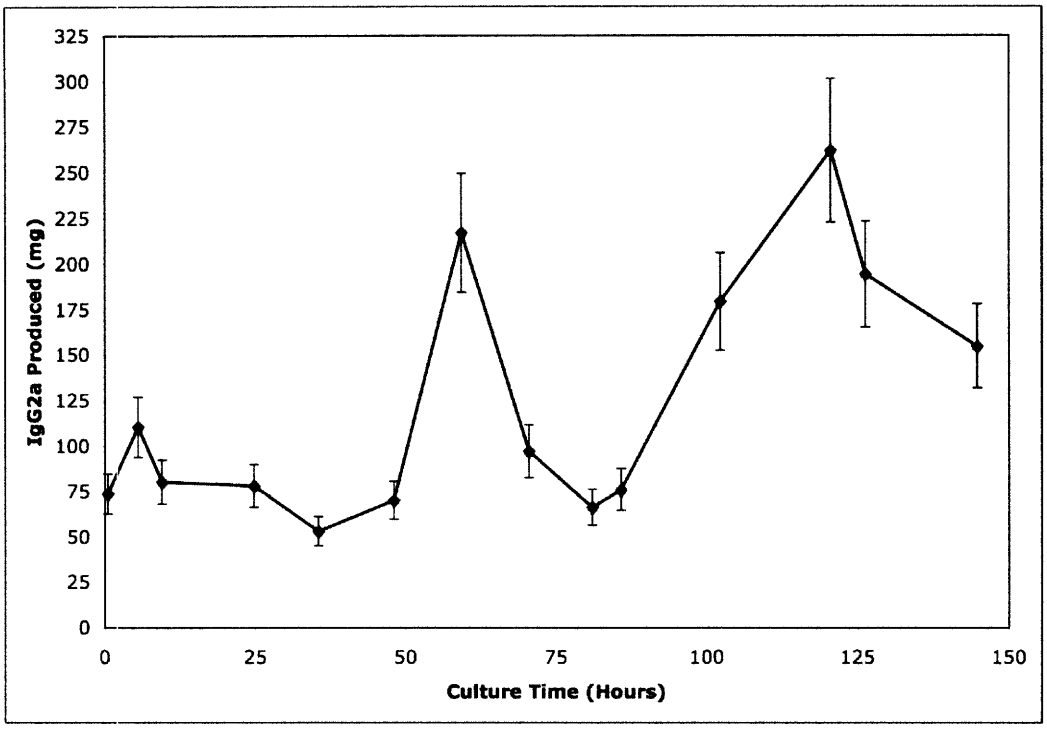

Figure 44 – Total Amount of Monoclonal Antibody Produced vs. Time for IB4 Control Batch Run. The error bars correspond to one standard deviation.

The total amount of monoclonal antibody produced by the IB4 batch culture containing the ceramic membrane module over time is shown in Figure 44. The qualitative trend illustrated by Figure 43 is also evident in Figure 44. Initially, there was a small amount of antibody produced in the reactor. The total amount of antibody increased from around 50 mg at 40 hours to nearly 225 mg at 60 hours. The amount of IgG_{2a} then decreased to around 50 mg by 80 hours. Another increase in antibody occurred after this time. The maximum amount of IgG_{2a} produced by the culture was 260 mg at 120 hours. The final amount of antibody present at the end of the culture was over 150 mg. Both the maximum amount and the final amount of monoclonal antibody observed in the reactor is much greater than that seen in either the batch or fed-batch reactors. Since Chinese hamster ovary cells tend to produce more monoclonal antibody when under stress, it is assumed that the presence of the ceramic membrane module in the reactor places a fluid dynamic stress on the CHO cells. Although similar cell concentrations and viabilities were seen in the batch culture containing the ceramic membrane module and in the batch culture not containing the ceramic membrane module,

the control batch experiment produced more monoclonal antibody per individual cell. Although the fed-batch experiment reached higher cell concentrations than the control batch experiment, the batch culture containing the ceramic membrane module also produced more total antibody than the fed-batch culture.

Table 7 – Actual Set of Perfusion Rates for SCMR Culture of IB4 Cells

Time	Perfusion Rate (Volume of Fresh Medium / Working volume / Day)
0 – 70 hours	0 vvd
70 – 78 hours	1.3 vvd
78 – 96 hours	0.6 vvd
96 – 120 hours	1.5 vvd
120 – 155 hours	2.5 vvd

Perfusion experiments were next performed to evaluate the performance of the SCMR system with the IB4 hybridoma cell line. The optimal perfusion feeding strategy was determined partly from previous research with this equipment (23-25) and partly from repeated experiments testing the equipment and feeding strategies on the hybridoma cells. The optimal set of feeding rates found by previous research (24) for perfusion culture of IB4 hybridoma cells in serum-free medium in a 1 liter working volume are shown in the previous section discussing the experiments with the HPCHO Chinese hamster ovary cell line in Table 2. The optimal set of feeding rates found for SCMR perfusion culture of IB4 hybridoma cells in BD Cell mAb Medium Quantum Yield are shown in Table 7. The two sets of perfusion rates do not match. It is important to note that every attempt was made to increase the perfusion rate at set stages, but there were several problems. At some point during the third time period (between 78 and 96 hours) the pump rate was decreased accidentally and the overall perfusion rate for that time was therefore greatly reduced. Towards the end of the SCMR run, there was a leak in the harvest bottle that may have affected the flow of the harvest out of the ceramic membrane module. Also, due to some fouling of the ceramic membrane module, the perfusion rates were sometimes lower than what had been expected.

The most successful SCMR experiment with the IB4 hybridoma cell line will be discussed. The three-liter bioreactor was inoculated with a 1.8-liter working volume of IB4 culture with an initial density of around 1.5×10^5 cells/mL. Disturbances included

the purging of the sample line of 3 mL and the sampling of 5 mL twice daily, as well as feeding of fresh medium and harvesting of spent medium after 70 hours. The autoclaved 3 liter bottles that contained the fresh medium to be fed and that collected the spent medium were replaced with a new set of autoclaved bottles after 130 hours. The total amount of medium supplied to the IB4 SCMR system was 8.1 liters.

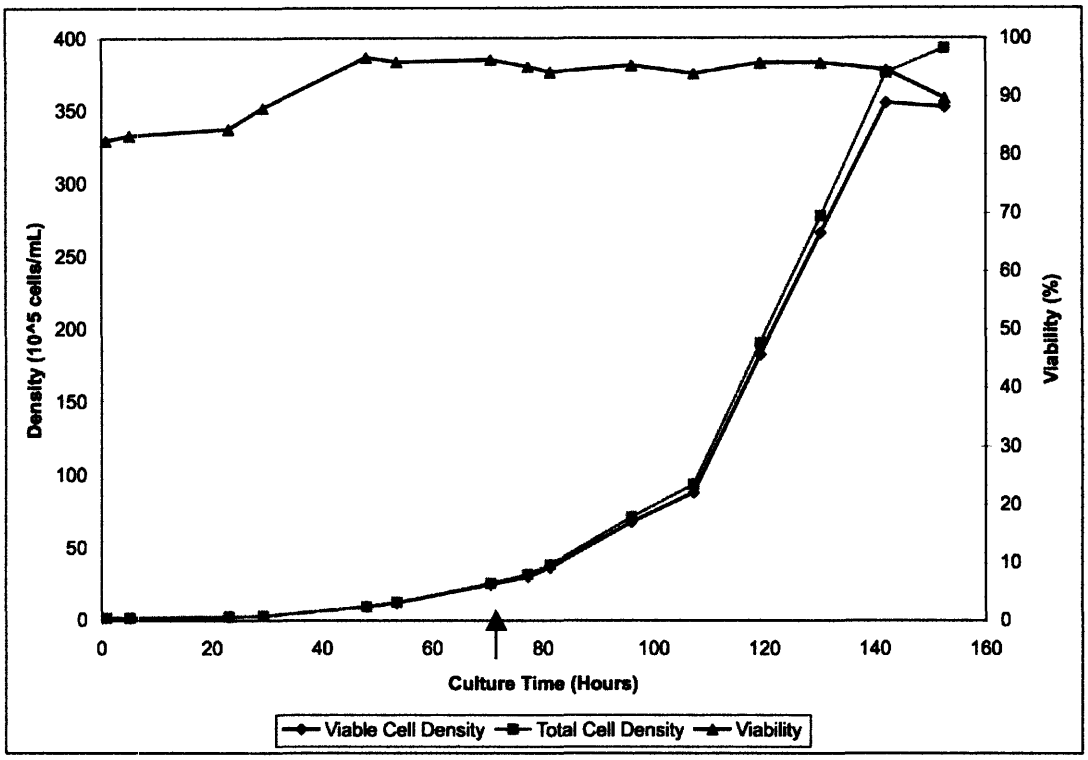

Figure 45 – Growth Data for IB4 SCMR Run. The black arrow indicates the start of perfusion.

The cell concentration and viability data for the IB4 hybridoma SCMR experiment are shown in Figure 45. A graph comparing the cell concentration data for both the batch and the SCMR hybridoma experiments is shown in Figure 46, so that the two experiments may be compared visually. The SCMR data series have solid lines and the batch data series have dashed lines. The SCMR culture experienced a lag phase until around 30 hours. The lag phase was due to the adjustment of the hybridoma cells to the new environment of the stirred tank reactor. Because this period was only 5 hours longer in length than the lag phase in the batch experiment, the observation that the presence of the ceramic membrane module had little to no significant adverse effect on the culture environment was reinforced.

Three interesting regions characterized the exponential growth phase of the SCMR IB4 culture. The first segment of the exponential growth phase began at 30 hours and continued until around 78 hours. Since perfusion was initiated at around 70 hours, the first segment of the exponential growth phase corresponds to the growth possible in batch conditions. The average growth rate during this period was 0.046/hr. The maximum growth rate during this period was 0.061/hr at about 50 hours. The average and maximum growth rates of this time period of the SCMR culture were quite similar to those for the batch IB4 culture, which were an average growth rate of 0.04/hr and the maximum growth rate was 0.063/hr. This further proves that the inclusion of the ceramic membrane module inside the bioreactor had little effect on the growth of the hybridoma cells. Feeding and harvesting of the bioreactor were initiated after 70 hours. The second exponential growth phase began around 78 hours and continued for a total of 30 hours. This time period corresponds to extended growth of the culture due to perfusion. The average growth rate during this period was 0.039/hr, which was slightly slower than that for the batch IB4 culture. The last segment of the exponential growth phase began around 108 hours and continued until the nearly the end of the culture, until 140 hours. This time period corresponded to continued growth of the culture, but at a slowed pace. Significant aggregation of the hybridoma cells also occurred around this time and clumps of cells were visible in the bioreactor during this third exponential growth phase. The average growth rate during this period was 0.036/hr. This average growth rate was much slower than that of the batch IB4 culture. This was most likely due to a decrease in nutrients that were available to the high density of hybridoma cells. The perfusion rates shown in Table 3 are evidence of this theory. At one point, the perfusion rate was accidentally decreased instead of increased, and overall, the perfusion rates were lower than what had been intended. This lower than desired perfusion rate allowed less nutrients to reach the high density of IB4 hybridoma cells than was desired. The various metabolite concentrations of the IB4 SCMR experiment will be examined in more detail below to verify these observations. After 140 hours, the hybridoma culture appeared to settle into a stationary phase at the maximum viable cell concentration reached by the experiment. Due to the aggregation of the hybridoma cells and the extensive fouling of the ceramic membrane module, the experiment was halted at about 155 hours when

medium was no longer harvested from the ceramic membrane module. Consequently, the stationary phase of the IB4 SCMR culture was interrupted and there was no death phase observed in the experiment.

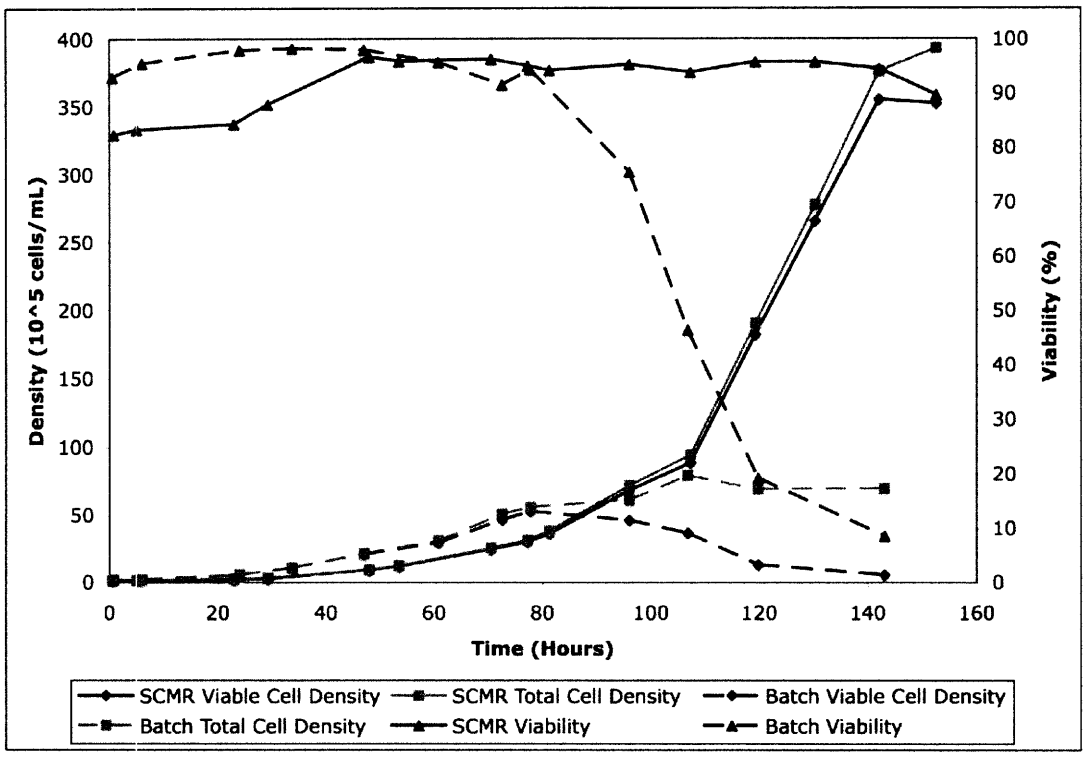

Figure 46 – Comparison of Growth Data for IB4 Batch Run and IB4 SCMR Run

The maximum viable cell concentration reached in the IB4 SCMR experiment was 355.47 x 10^5 cells/mL at about 140 hours, the end of the exponential growth phase of the culture. As Figure 46 shows, this value was over seven times that achieved in the IB4 batch experiment. The maximum viable cell concentration in the SCMR experiment was also over seven times that achieved in the control batch culture and nearly six times that reached by the fed-batch experiment. The maximum total cell concentration achieved was 393.13 x 10^5 cells/mL, at the end of the culture at 155 hours. As Figure 46 shows, this value was over four times that achieved in the IB4 batch experiment. The maximum total cell concentration in the SCMR experiment was also over four times that achieved in both the control batch and fed-batch hybridoma cultures. The very high viable and total cell concentrations achieved in this IB4 SCMR experiment were similar to the results found in a study involving IB4 hybridoma cells grown in serum-free medium with the SCMR system (24). Excellent monoclonal antibody productivities were also found in

that study. As will be discussed below, the high concentrations of hybridoma cells should have led to the production of a large amount of IgG_{2a} antibody.

The viability of the SCMR culture remained high throughout the experiment. Although the viability of the IB4 cells at inoculation was slightly low (around 83%), the viability of the culture quickly increased to over 95% by 48 hours. The viability of the cells remained above 93% until after 140 hours and above 89% until the very end of the experiment. This showed that perfusion involving the ceramic membrane module had little to no adverse effect on the viability of the IB4 hybridoma cells. Taken together, this data showed that the feeding of fresh medium and the harvesting of spent medium allowed the IB4 culture to reach much higher cell concentrations while maintaining a high viability of the culture. This combination should have had an excellent effect on the monoclonal antibody productivity of the system.

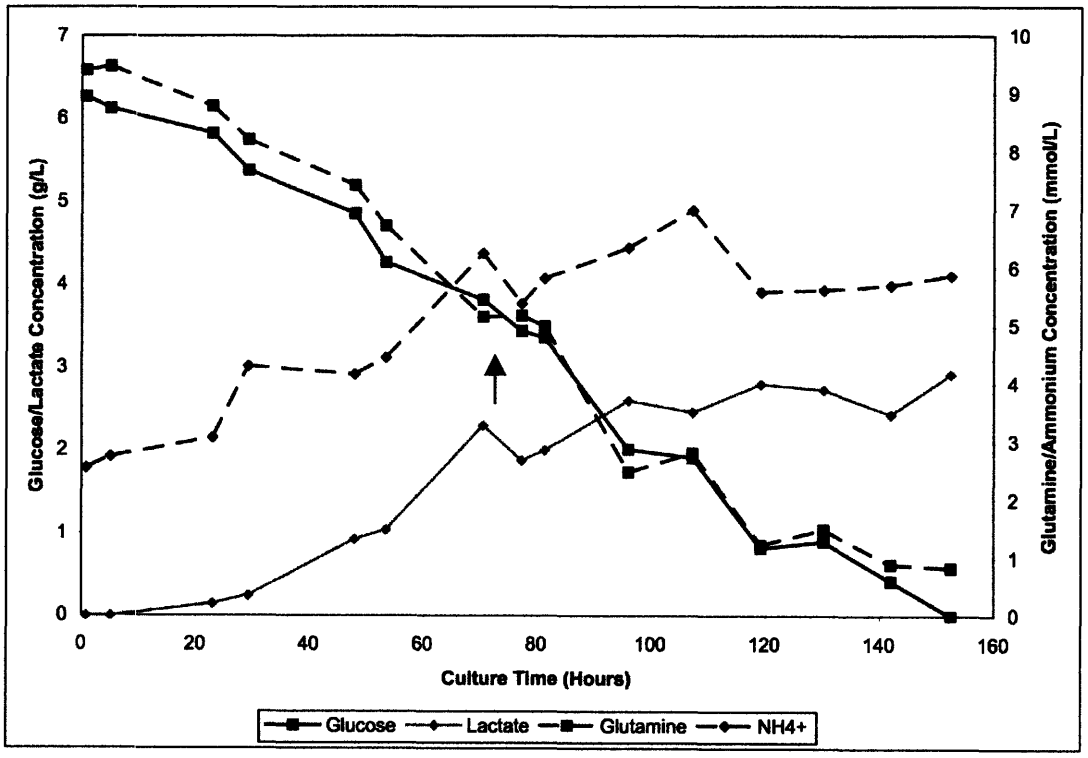

Figure 47 – Metabolic Data for IB4 SCMR Run. The black arrow indicates the start of perfusion.

Important metabolite concentrations for the SCMR hybridoma experiment are shown in Figure 47. At inoculation, the glucose concentration was over 6.25 g/L. The concentration of glucose decreased steadily until it was about 3.8 g/L after 70 hours. Feeding of fresh medium and harvesting of spent medium was initiated at this time. A

series of plateaus and declines in glucose concentration then occurred. Every time the perfusion rate was increased, the concentration of glucose would be maintained for a short period of less than 10 hours. After this short period, the high density of hybridoma cells would begin consuming glucose at a faster rate than it could be supplied. When the experiment was halted due to the high density of cells causing aggregation and eventually fouling of the ceramic membrane module, the glucose concentration had finally decreased to 0 g/L. At inoculation, the concentration of lactate in the SCMR run was 0 g/L. During the batch segment of the experiment, the lactate concentration steadily increased until it reached 2.3 g/L at 70 hours. This value corresponded to the amount of glucose that was taken up by the hybridoma cells, so it may be assumed that during this phase, the IB4 culture utilized glucose mainly for growth and cellular metabolism. Perfusion of the SCMR system was initiated after 70 hours. Because the concentration of lactate initially dropped from 2.3 g/L at 70 hours to under 1.9 g/L at 78 hours, it is apparent that the ceramic membrane module was effective at removing this product of cellular metabolism from the reactor. As the glucose concentration continued to slowly decrease due to the high density of cells utilizing more and more of this nutrient, however, the lactate concentration increased very slightly to around 2.6 g/L by 96 hours. For the remainder of the experiment, the concentration of lactate continued to oscillate between 2.4 and 2.9 g/L. Since the ceramic membrane was quite effective at removing excess lactate from the reactor, it was impossible to determine the degree of glucose utilization for the production of monoclonal antibody from the metabolic concentration data. The results from the batch culture containing the ceramic membrane module, however, predict a very high production of monoclonal antibody.

At inoculation, the glutamine concentration of the SCMR hybridoma culture was nearly 9.5 mmol/L. The concentration of this nutrient declined steadily over time until it was under 5.2 mmol/L at 70 hours. Feeding of fresh medium and harvesting of spent medium from the SCMR system was initiated at this time. Initially, perfusion maintained the glutamine concentration above 5 mmol/L for 10 hours. When the perfusion rate was accidentally set too low, however, the hybridoma cells began consuming more glutamine than could be replaced. The concentration of glutamine was thus about 2.5 mmol/L by 96 hours. As was discussed above concerning the glucose concentration, there were

plateaus and declines in glutamine concentration for the remainder of the experiment dependent on increases in perfusion rate. When the experiment was halted due to clogging, the glutamine concentration was maintaining a value of 0.8 mmol/L and it appeared that glucose was being utilized preferentially by the cells. At inoculation, the concentration of ammonium ion in the IB4 SCMR experiment was over 2.5 mmol/L. During the batch segment of the experiment, the ammonium ion concentration steadily increased as glutamine was consumed by the cells. By 70 hours, the ammonium ion concentration was about 6.25 mmol/L. Perfusion was initiated at this time. The concentration of ammonium ion dropped immediately after perfusion of the system began to under 5.4 mmol/L. This showed that the ceramic membrane module was effective at removing this toxic waste product from the culture, allowing greater glucose utilization by the IB4 hybridoma cells. The ammonium ion concentration later increased to 7 mmol/L by 108 hours, but then remained below 6 mmol/L from 120 hours to the end of the culture due to the cells primarily using glucose rather than glutamine.

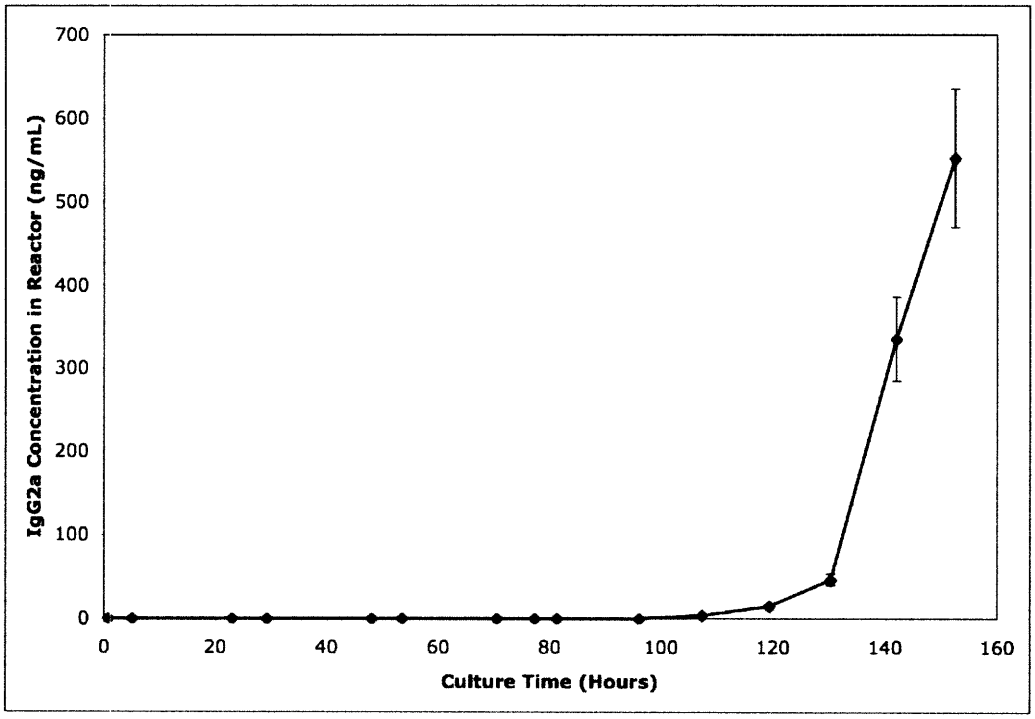

Figure 48 – Monoclonal Antibody Concentration Inside Reactor vs. Time for IB4 SCMR Run. The error bars correspond to one standard deviation.

The IgG_{2a} monoclonal antibody concentration in the IB4 SCMR culture was determined periodically. The capillary electrophoresis data illustrating the product

concentration of the hybridoma SCMR culture over time is shown in Figure 48. There were no detectable levels of antibody while the hybridoma cells were in the lag phase and the early stages of the exponential growth phase. The concentration of IgG_{2a} monoclonal antibody in the SCMR culture increased quite rapidly in the final stages of the exponential growth phase and the beginning of an apparent stationary phase. At 120 hours, the product concentration was about 15 ng/mL. The final concentration of antibody in the reactor at 155 hours was over 550 ng/mL. This surge in product concentration corresponds to both the slowed growth of the hybridoma cells and the period in which glucose rather than glutamine was preferentially taken up by the hybridoma cells. The final antibody concentration in the SCMR experiment was about seven times that achieved in the batch run and six times that achieved in the fed-batch and control batch experiments.

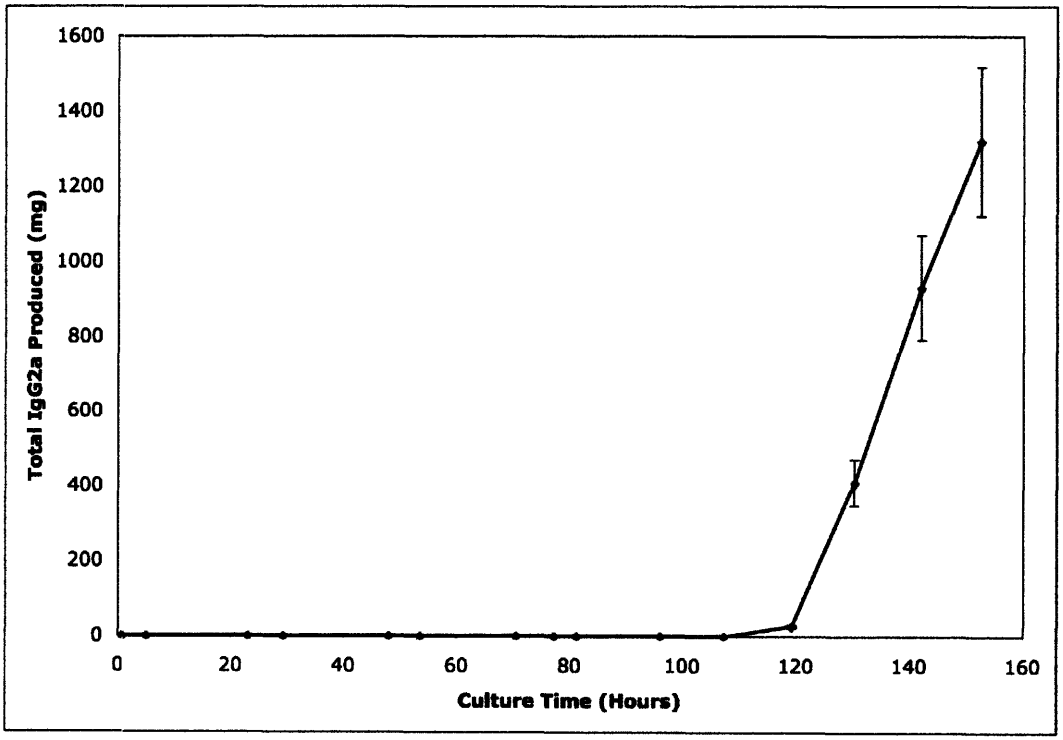

Figure 49 – Total Monoclonal Antibody Production vs. Time for IB4 SCMR Run. The error bars correspond to one standard deviation.

The total amount of monoclonal antibody produced by the IB4 SCMR culture over time is shown in Figure 49. The qualitative trend illustrated by Figure 48 is also evident in Figure 49. Initially, there was no detectable amount of antibody produced in

the reactor. By 120 hours, around 25 mg of antibody had been produced by the system. After this point, the amount of IgG_{2a} produced by the system increased quite rapidly. By the time the experiment was halted due to fouling of the ceramic membrane module, about 1.32 g of monoclonal antibody had been produced by the system. Although about 325 mg of monoclonal antibody was harvested from the reactor between 108 and 130 hours, the remaining amount of antibody was recovered from the reactor volume. This implies that it was difficult for the monoclonal antibody to perfuse through the ceramic membrane module. The total amount of antibody produced by the SCMR system was an order of magnitude greater than the amounts of monoclonal antibody produced in the batch, fed-batch, and control batch cultures. This amount was significantly less than the 8.06 g previously produced using serum-free medium with the IB4 SCMR system (24). Switching to a serum-free medium composition, therefore, would provide even better results. It should also be noted that the glycosylation of the product isolated from this system may be different from that isolated from the batch system because different feeding strategies may effect the glycosylation of mammalian cell products (33). The effects on glycosylation caused by feeding strategies may be enhanced in this case since the ceramic membrane module was not effective in removing much of the product from the reactor volume and the monoclonal antibody may have degraded or been altered in some other fashion. Even when degradation effects are considered, however, the IB4 SCMR system outperformed the batch and fed-batch systems in maximum cell concentration, viability, and monoclonal antibody production.

4.4 Alternating tangential flow hollow fiber system

Several perfusion experiments involving the alternating tangential flow (ATF) hollow fiber membrane system were performed with the IB4 hybridoma cell line as described above. The same ATF controller settings used in the ATF experiments with the HPCHO cell line were employed in these experiments. The pressure set point of the ATF controller was about 4 psi and the exhaust cycle lasted approximately 10 seconds. The optimal perfusion feeding strategy was determined partly from previous research involving growing the IB4 hybridoma cells with other equipment such as the SCMR

system and partly from repeated experiments testing the ATF equipment and feeding strategies on the IB4 cells. The optimal set of feeding rates found for ATF perfusion culture of IB4 hybridoma cells in BD Cell mAb Medium Quantum Yield are shown in Table 8. These perfusion rates were not the same as the perfusion rates used in the SCMR experiment. It is important to note that every attempt was made to increase the perfusion rate at set stages, but the degree of fouling of the hollow fiber membrane increased over time and many of the perfusion rates were lower than what had been anticipated.

Table 8 – Actual Set of Perfusion Rates for ATF Culture of IB4 Cells

Time	Perfusion Rate (Volume of Fresh Medium / Working volume / Day)
0 – 72 hours	0 vvd
72 – 78 hours	1.2 vvd
78 – 96 hours	1.3 vvd
96 – 108 hours	1.2 vvd
108 – 120 hours	2 vvd

The most successful ATF experiment with the IB4 hybridoma cell line will be discussed. The three-liter bioreactor was inoculated with a 1.8-liter working volume of IB4 culture with an initial density of around 1.5×10^5 cells/mL. Disturbances included the purging of the sample line of 3 mL and the sampling of 5 mL twice daily, as well as feeding of fresh medium and harvesting of spent medium after 72 hours. The autoclaved 3 liter bottles that contained the fresh medium to be fed and that collected the spent medium were replaced with a new set of autoclaved bottles after 107 hours. The total amount of medium supplied to the IB4 ATF system was 7.4 liters.

The cell concentration and viability data for the IB4 ATF experiment are shown in Figure 50. A graph illustrating the cell concentration and viability data for both the batch and the ATF IB4 hybridoma experiments is shown in Figure 51, so that the two experiments may be compared visually. The ATF data series have solid lines and the batch data series have dashed lines. Figure 52 contains a graph comparing the cell concentration and viability data for the ATF and SCMR IB4 hybridoma experiments. The ATF data series again have solid lines and the SCMR data series have dashed lines. The ATF hybridoma culture experienced a quite short lag phase of slightly less than 20

hours. The lag phase was due to the adjustment of the hybridoma cells to the new environment of the stirred tank reactor. Because this period was similar in length and actually slightly shorter than the lag phases in both the batch and SCMR experiments, it was concluded that the ATF equipment set-up had little to no effect on the growth and viability of the IB4 hybridoma cells. This was to be expected since the ATF system is primarily an external perfusion system.

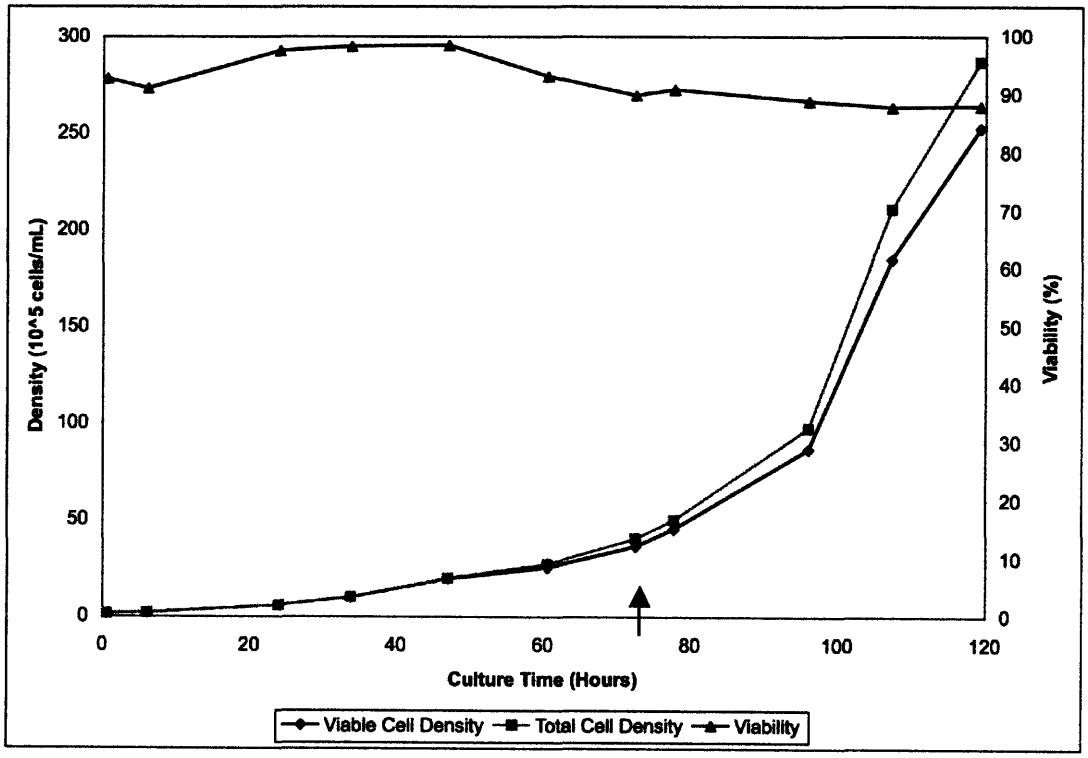

Figure 50 – Growth Data for IB4 ATF Run. The black arrow indicates the start of perfusion.

The exponential growth phase of the IB4 ATF culture was quite interesting. The average growth rate over the entire exponential growth phase (from 20 hours to 120 hours) was 0.042/hr. This was slightly faster than the growth rate of the batch culture of 0.04/hr but slightly slower than the growth rate of the first exponential growth phase of the SCMR culture of 0.046/hr. However, the point growth rates varied throughout the experiment. From 20 hours to 60 hours, the average growth rate was 0.054/hr. From 60 hours to the end of the culture at 120 hours, the average growth rate was 0.037/hr, but this included a maximum growth rate of 0.067/hr at 110 hours. It appeared that the cells took some time to adjust to the perfusion that was initiated after 72 hours. By 110 hours, the cells had adjusted to the shear forces generated by the ATF cycling and the growth rates

of the hybridoma culture increased. The exponential growth phase of the ATF culture was cut short, however. Because the experiment was halted after 120 hours when the ATF hollow fiber membrane was clogged, the hybridoma culture also experienced no stationary phase and no death phase.

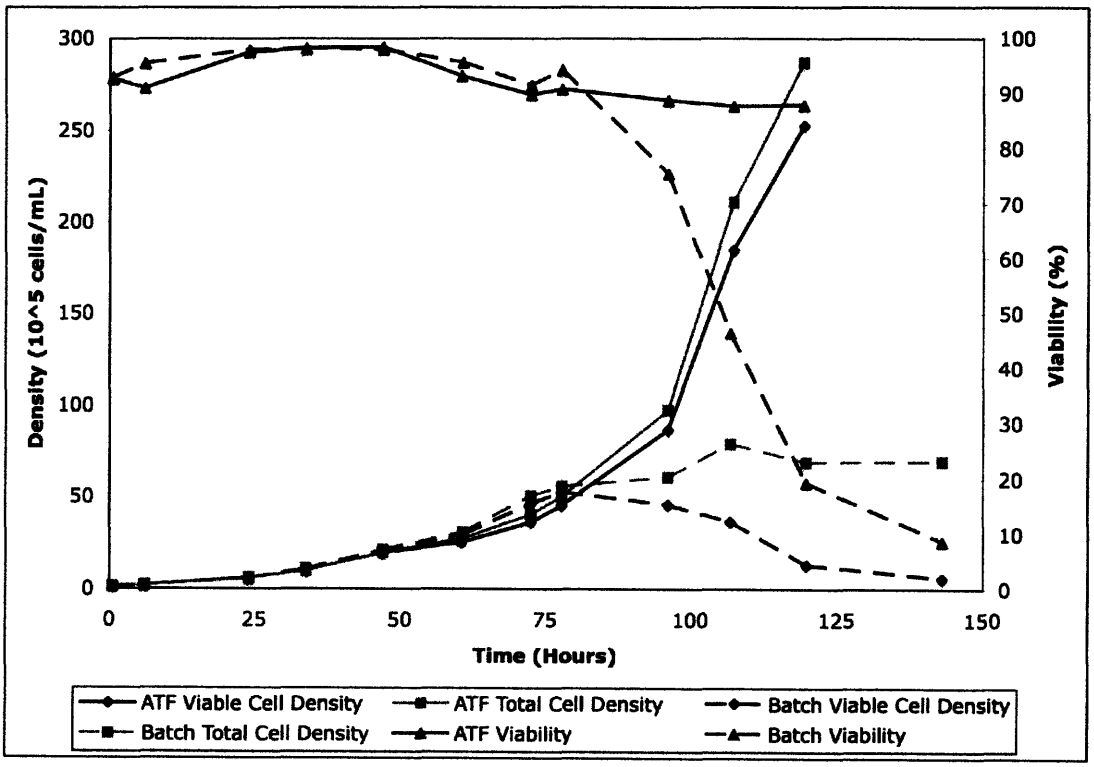

Figure 51 – Comparison of Growth Data for IB4 Batch Run and IB4 ATF Run

The maximum viable cell density achieved by the IB4 ATF culture was 252.62 x 10^5 cells/mL at 120 hours, the end of the experiment. This value was about five times that achieved in the IB4 batch culture and over four times that achieved in the IB4 fed-batch culture. As shown in Figure 52, this value was much lower than the maximum viable cell concentration reached in the IB4 SCMR culture of 355.47 x 10^5 cells/mL after 140 hours. The maximum total cell concentration achieved by the IB4 ATF culture was 287.03 x 10^5 cells/mL. This value was about 3.5 times that achieved in the IB4 batch and fed-batch experiments. As shown in Figure 52, this value was much lower than the maximum total cell concentration reached in the IB4 SCMR culture of 393.13 x 10^5 cells/mL at 155 hours. It can be concluded that the ATF experiment was more effective in growing the hybridoma cells to high densities than the batch or fed-batch experiments, but was less effective than the SCMR experiment. As will be discussed below, the high

concentrations of hybridoma cells in the ATF experiment should also have led to the production of a large amount of IgG_{2a} antibody.

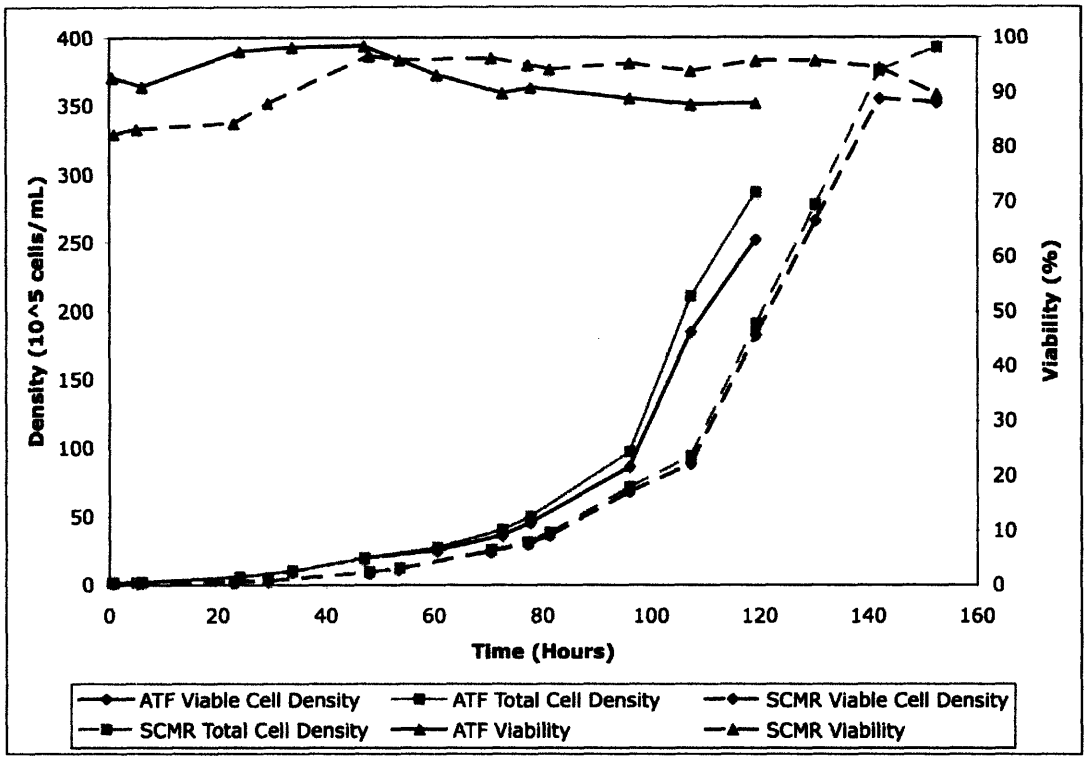

Figure 52 – Comparison of Growth Data for IB4 SCMR Run and IB4 ATF Run

As was observed in the SCMR experiment, the viability of the IB4 cells in the ATF experiment at inoculation was slightly low (90-93%). During the batch segment of the ATF culture, the viability quickly increased to over 95% by 24 hours. After 48 hours, the viability of the cells began to drop until it was below 90% by 72 hours. Feeding and harvesting of the reactor was initiated at this time. As the cycling of the ATF system proceeded, the viability of the culture continued to slowly decline. When the experiment was halted after 120 hours due to fouling of the hollow fiber membrane cartridge, the viability of the ATF culture was 88%. This showed that perfusion involving the ATF hollow fiber membrane system had a negative effect on the viability of the IB4 hybridoma cells. This negative effect was not nearly as strong as that seen in the ATF experiment with the HPCHO Chinese hamster ovary cells, however. Taken together, this data showed that the feeding of fresh medium and the harvesting of spent medium allowed the IB4 culture to reach much higher cell concentrations, but the shear forces

generated by the alternating tangential flow through the hollow fiber membrane decreased the viability of the culture.

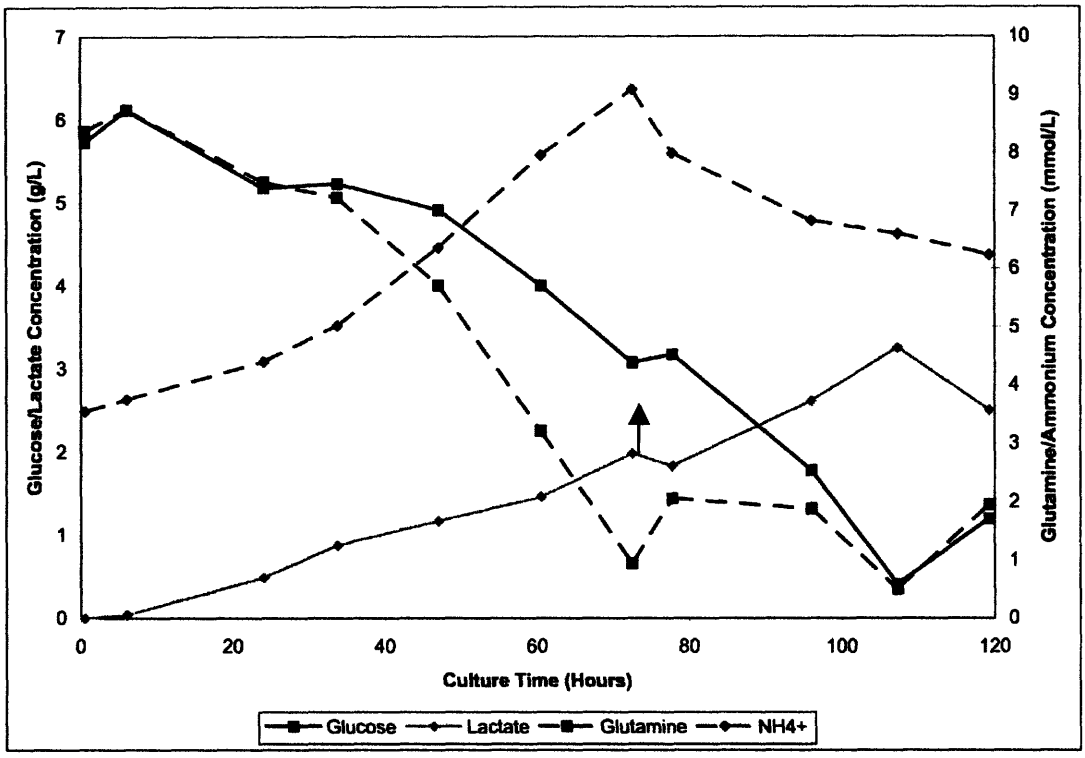

Figure 53 – Metabolic Data for IB4 ATF Run. The black arrow indicates the start of perfusion.

Important metabolite concentrations for the ATF IB4 hybridoma experiment are shown in Figure 53. At inoculation, the glucose concentration was about 6 g/L. The concentration of this nutrient decreased slowly until it was about 3 g/L at 72 hours. After perfusion was initiated, the glucose concentration increased slightly to 3.2 g/L at 78 hours. Although the speed of the peristaltic pump was increased at this time, the perfusion rate remained the same. As a result, the glucose concentration decreased until it was 0.4 g/L at 108 hours. The perfusion rate was increased from 1.2 vvd to 2 vvd at this time. As a result, the concentration of glucose in the culture increased to 1.2 g/L by the end of the culture. There was no lactate in the culture in the ATF system at inoculation. After inoculation, the concentration of lactate in the culture slowly increased until it was about 2 g/L at 72 hours. The difference between the amount of glucose consumed by the hybridoma cells and the amount of lactate produced by the cells was nearly 1 g/L. The IB4 cells were probably utilizing some of the glucose for the production of monoclonal antibody rather than cellular metabolism. It is interesting to

note that this phenomenon was not apparent in the SCMR reactor and antibody production was not seen in that case until perfusion had been initiated. Feeding of fresh medium and harvesting of spent medium from the ATF system was initiated after 72 hours. The ATF system was initially successful at removing excess lactate from the IB4 culture. The lactate concentration in the reactor decreased to 1.8 g/L by 78 hours. The perfusion rate remained about the same, however, for the next 30 hours. As a result, the concentration of lactate in the culture slowly increased to 3.25 g/L by 108 hours. When the perfusion rate increased from 1.2 vvd to 2 vvd at 108 hours, the concentration of lactate began decreasing again. The final concentration of lactate in the ATF culture was 2.5 g/L.

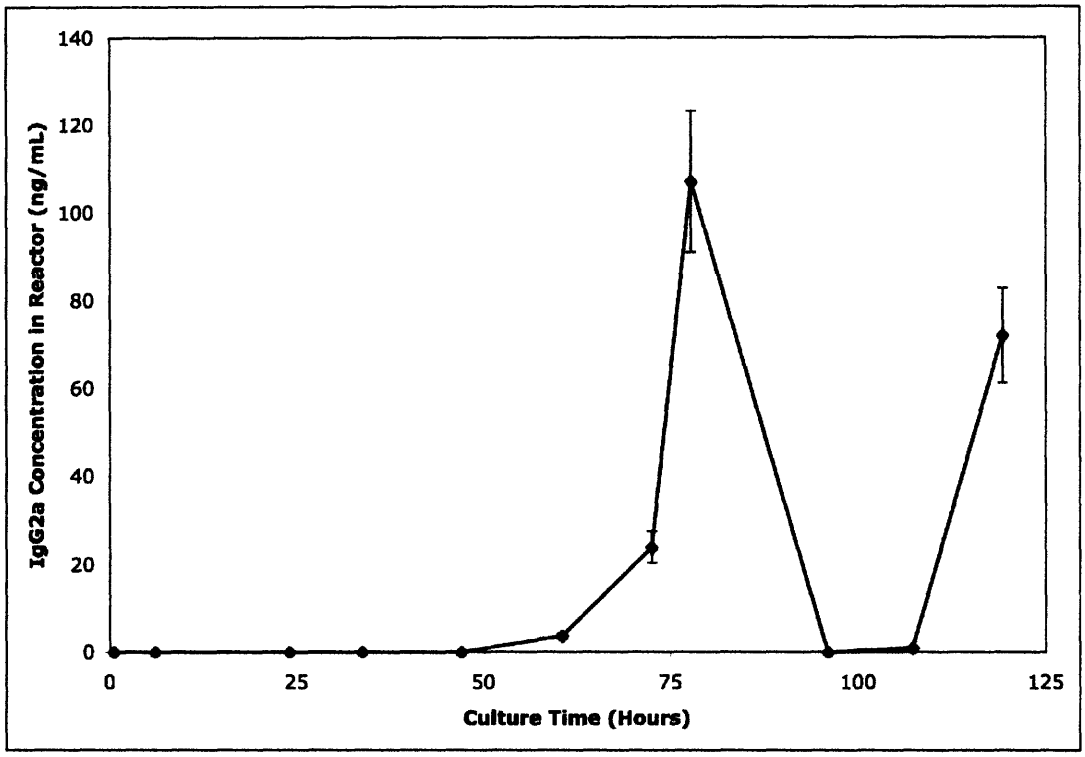

Figure 54 – Monoclonal Antibody Concentration Inside Reactor vs. Time for IB4 ATF Run. The error bars correspond to one standard deviation.

At inoculation, the glutamine concentration of the IB4 ATF experiment was about 8.5 mmol/L. During the batch phase of the experiment, the glutamine concentration decreased quickly to less than 1 mmol/L by 72 hours. ATF cycling began at this point. Perfusion of the system initially doubled the glutamine concentration to 2 mmol/L by 78 hours. With some adjustment of the perfusion rate, this average concentration was

maintained for the remainder of the experiment. As seen in the IB4 SCMR experiment, less glutamine consumption than glucose consumption was observed towards the end of the exponential growth phase. This trend validated the idea that the hybridoma cells began producing more monoclonal antibody towards the end of the experiment. The initial concentration of ammonium ion in the ATF system was about 3.5 mmol/L. The concentration of this toxic metabolite increased quickly to over 9 mmol/L by 72 hours. The ATF system was quite successful at removing ammonium ion from the culture, however. After perfusion was initiated at 72 hours, the ammonium ion concentration decreased steadily to a final value of 6.25 mmol/L. Removal of this toxic waste product helped the hybridoma cells continue to take up glucose to use for cellular metabolism purposes as well as monoclonal antibody production.

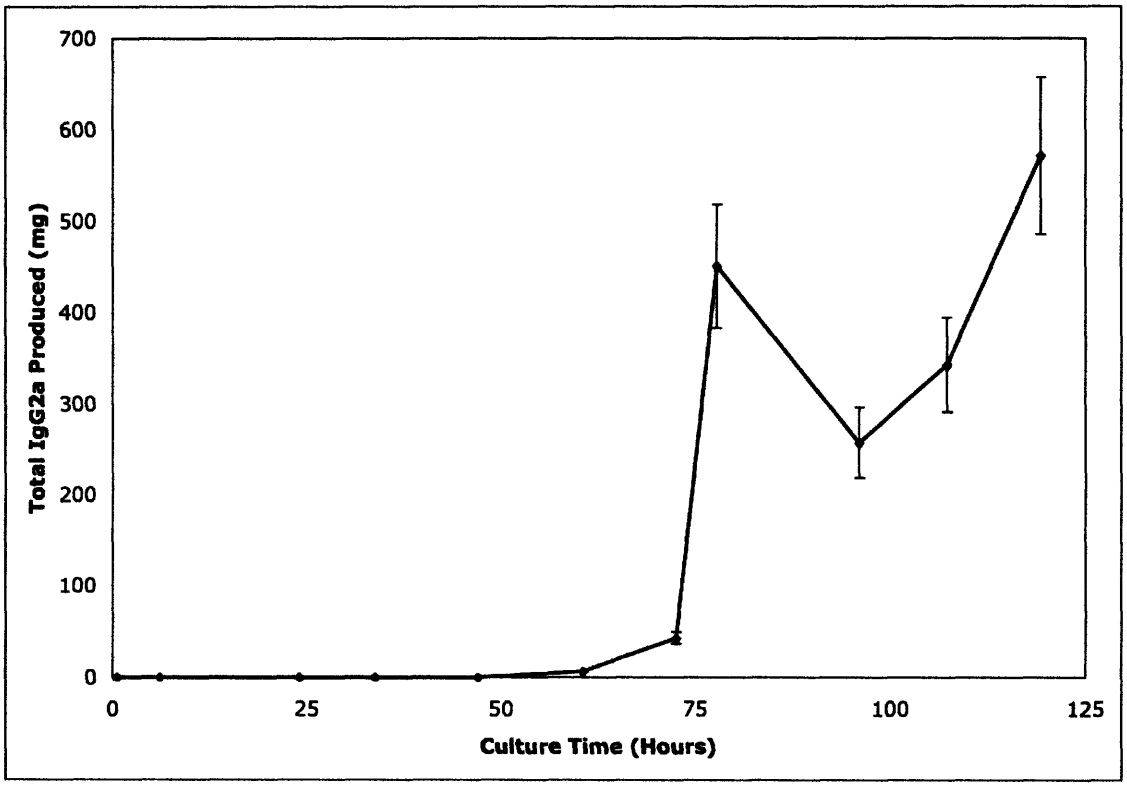

Figure 55 – Total Monoclonal Antibody Produced vs. Time for IB4 ATF Run. The error bars correspond to one standard deviation.

The IgG_{2a} monoclonal antibody concentration in the IB4 ATF experiment was determined periodically. The capillary electrophoresis data illustrating the product concentration of the hybridoma ATF culture over time is shown in Figure 54. There were no detectable levels of antibody while the hybridoma cells were in the lag phase and the

early stages of the exponential growth phase. The concentration of IgG_{2a} monoclonal antibody in the ESF culture increased during the later stages of the exponential growth phase. At 60 hours, the concentration of monoclonal antibody in the ATF culture was 3.7 ng/mL. The product concentration continued to increase until it was 107 ng/mL at 78 hours. This was shortly after perfusion of the ATF system was initiated. The monoclonal antibody concentration in the reactor then decreases, most likely due to antibody being harvested from the system. By 96 hours, there were no detectable levels of antibody remaining in the reactor. After 108 hours, the product concentration in the bioreactor began to increase until it was over 72 ng/mL at the end of the experiment. This increase in monoclonal antibody production corresponded to the rapid increase in cell density and the increased dependence of the culture on glucose. The concentrations of monoclonal antibody found in the ATF reactor during this experiment were not significantly different than the product concentrations observed in the batch and fed-batch experiments. They were also much lower than the product concentrations observed in the SCMR experiment. However, the production of monoclonal antibody by the IB4 hybridoma cells apparently began at an earlier point in time in the ATF culture than it did in the SCMR culture.

The total amount of monoclonal antibody produced by the IB4 ATF culture over time is shown in Figure 55. The qualitative trend illustrated by Figure 54 is evident but slightly altered in Figure 55. Initially, there was no detectable amount of antibody produced by the system. By 60 hours, a total of 6.6 mg of IgG_{2a} had been produced. After perfusion began at 72 hours, the amount of antibody produced increased quickly to over 450 mg by 78 hours. After this time, a decrease in the total amount of antibody produced was seen. At 96 hours, about 250 mg of antibody had been produced. This may have been due to error, but it was not as drastic as the decrease seen in Figure 54, because antibody was continually removed in the harvest stream. After 96 hours, the total amount of antibody produced by the system increased to a final value of over 570 mg. Although much less monoclonal antibody was produced by the ATF system than the SCMR system, much more monoclonal antibody was harvested from the ATF system than from the SCMR system. A total of 440 mg of antibody was collected from the harvest stream of the IB4 ATF experiment. Although the SCMR system produced a

greater total amount of monoclonal antibody, the ATF system allowed access to the product much earlier.

4.5 External spin filter system

Several perfusion experiments involving the external spin filter (ESF) system were performed with the IB4 hybridoma cell line as described above. The first goal of experimentation with the ESF equipment was to determine the optimal spin filter settings for perfusion of IB4 cells. The rotational speed of the spin filter module was first tested. The first value investigated was 100 rpm. Since this was the rotational speed of the impeller of the bioreactor, the tests performed at 100 rpm were what could have been expected from an internal spin filter. Much like the HPCHO Chinese hamster ovary cells discussed previously, the IB4 hybridoma cells were not well retained at this speed. Different rotational speeds of the spin filter were then tested, from 150 to 450 rpm. According to the manual for the spin filter apparatus, turbulent flow inside the apparatus began at rotational speeds of about 300 rpm. At speeds between 275 and 300 rpm, greater retention of cells was observed, but at higher speeds, the viability of the culture decreased. An initial rotation speed of 280 rpm was therefore chosen for the ESF perfusion run. After 25 hours of perfusion, the rotation speed of the spin filter module would be increased to 300 rpm. The recirculation rate of the IB4 culture was then tested. According to the manual for the spin filter apparatus, the recirculation rate must be kept at least at twice the speed of the perfusion rate to ensure proper recirculation of the culture. For low perfusion rates (1.5 vvd or lower), a recirculation rate of 150 rpm was found to be the most appropriate for the IB4 culture. For higher perfusion rates (1.5 vvd or above), a recirculation rate of 200 rpm was found to be the most appropriate for the IB4 culture. In any case of slowed perfusion due to aggregation of cells, it was found that increasing the recirculation rate above 200 rpm to about 220 rpm could improve the movement of cell aggregates through the tubing of the system and delay failure of the system due to fouling of the spin filter module. These results were quite similar to those attained for the HPCHO Chinese hamster ovary cell experiments involving the ESF system. From information gathered from the previous perfusion experiments with the

hybridoma cells, minimal testing of perfusion rates was needed. The optimal set of feeding rates found for ESF perfusion culture of IB4 hybridoma cells in BD mAb Quantum Yield Medium (BD Biosciences, MD) is shown in Table 9. The perfusion rates for the ESF experiment do not exactly match the perfusion rates for the SCMR or ATF experiments. It is important to note that every attempt was made to significantly increase the perfusion rate at set stages, but due to aggregation of the hybridoma cells as well as fouling of the steel external spin filter module, the perfusion rates were often lower than what had been expected.

Table 9 – Actual Set of Perfusion Rates and Spin Filter Rotation Speeds for ESF Culture of IB4 Cells

Time	Perfusion Rate (Volume of Fresh Medium / Working volume / Day)	Spin Filter Rotation Speed
0 – 62 hours	0 vvd	0 rpm
62 – 68 hours	0.9 vvd	280 rpm
68 – 85 hours	1.2 vvd	280 rpm
85 – 109 hours	1.2 vvd	300 rpm
109 – 120 hours	1.5 vvd	300 rpm
120 – 132 hours	1.8 vvd	300 rpm
132 – 140 hours	1.9 vvd	300 rpm

The most successful ESF experiment completed with the IB4 hybridoma cell line will be discussed. The three-liter bioreactor was inoculated with a 1.8-liter working volume of IB4 culture with an initial density of around 1.5×10^5 cells/mL. Disturbances included the purging of the sample line of 3 mL and the sampling of 5 mL twice daily, as well as feeding of fresh medium and harvesting of spent medium after 62 hours. The autoclaved 3 liter bottles that contained the fresh medium to be fed and that collected the spent medium were replaced with a new set of autoclaved bottles twice, after 90 and 120 hours. The total amount of medium supplied to the HPCHO ESF experiment was 9.6 liters.

The cell concentration and viability data for the IB4 ESF experiment are shown in Figure 56. A graph illustrating the cell concentration and viability data for both the batch and the ESF IB4 hybridoma experiments is shown in Figure 57, so that the two experiments may be compared visually. The ESF data series have solid lines and the

batch data series have dashed lines. Figure 58 contains a graph comparing the cell concentration and viability data for the ESF and SCMR IB4 hybridoma experiments. The ESF data series again have solid lines and the SCMR data series have dashed lines. The ESF culture experienced a lag phase until around 25 hours. The lag phase was due to the adjustment of the IB4 hybridoma cells to the new environment of the stirred tank reactor. Because this period was similar in length to the lag phases in the batch, SCMR, and ATF experiments, it was concluded that the ESF equipment set-up had little to no initial effect on the growth and viability of the hybridoma cells. This was to be expected since the ESF system is primarily an external perfusion system.

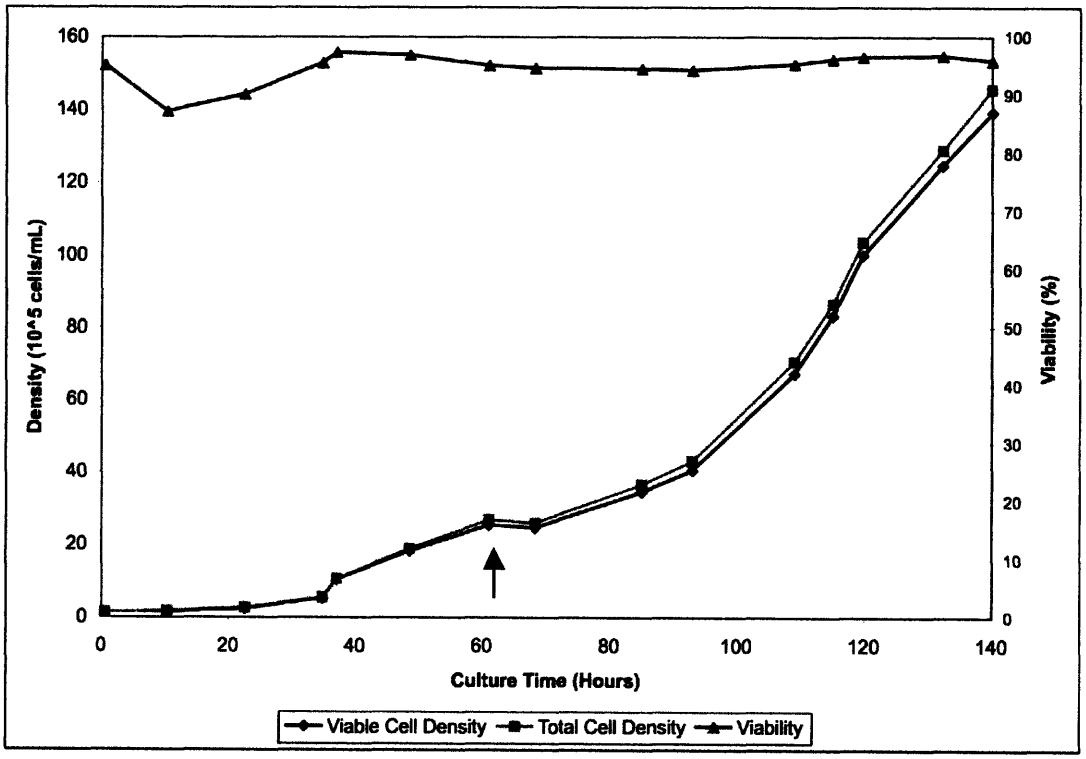

Figure 56 – Growth Data for IB4 ESF Run. The black arrow indicates the start of perfusion.

The exponential growth phase of the IB4 ESF experiment began at around 25 hours and continued until the end of the culture at 140 hours. The average growth rate for this time period was 0.051/hr. This average growth rate was the highest of the exponential growth rates for the three types of perfusion experiments, but the point growth rates varied widely for this time period. The lowest growth rate for the time period was 0/hr and the fastest growth rate for the time period was 0.30/hr. Because the IB4 ESF experiment was halted after 140 hours due to cell aggregation and fouling of the

steel spin filter module, the ESF culture experienced no stationary phase and no death phase.

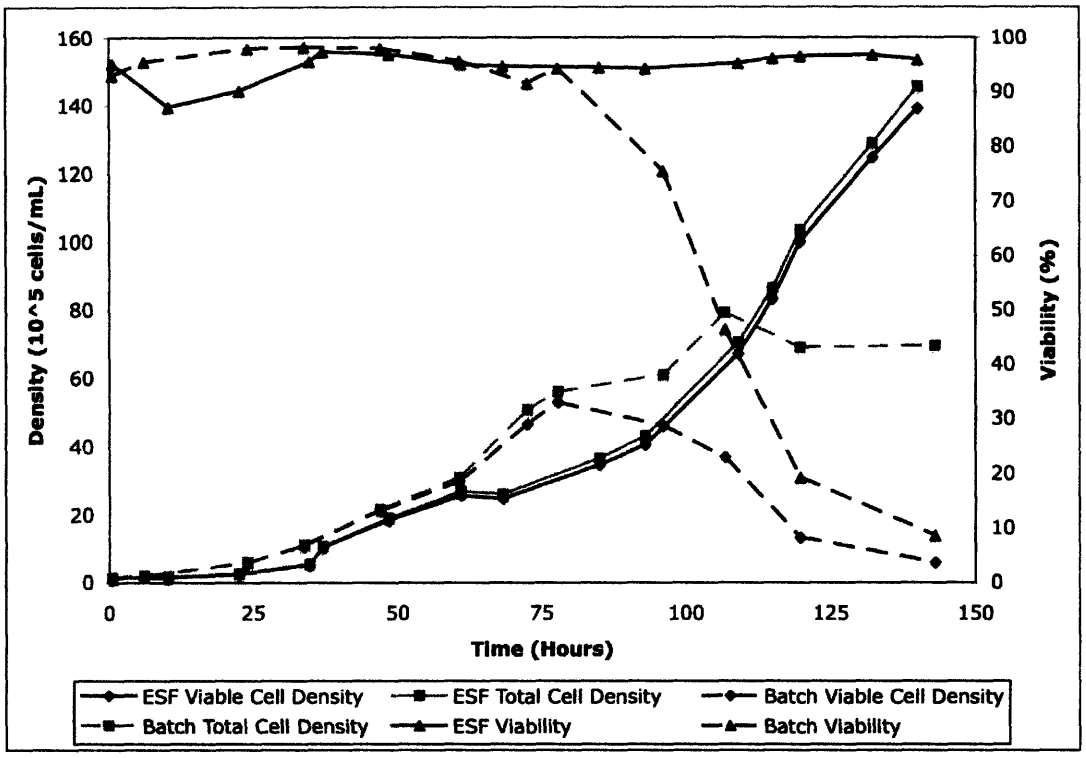

Figure 57 – Comparison of Growth Data for IB4 Batch Run and IB4 ESF Run

The maximum viable cell concentration reached in the IB4 hybridoma ESF experiment was 139.28 x 10^5 cells/mL at 140 hours. As Figure 57 shows, this value was much greater than the maximum viable cell concentration reached in the IB4 batch experiment. This value was also greater than the corresponding values in the fed-batch experiments. As Figure 58 shows, the maximum viable cell concentration reached in the IB4 ESF experiment was significantly less than that achieved in the SCMR experiment. This value was also less than that achieved in the ATF experiment. The maximum total cell concentration reached in the IB4 hybridoma ESF experiment was 145.64 x 10^5 cells/mL at 140 hours. Figures 57 and 58 show the same qualitative comparisons in maximum total cell concentrations for the different feeding strategies as in maximum viable cell concentrations.

During the ESF experiments with the hybridoma cells, it was found that a detectable proportion of cells continued to pass through the steel filter module and appeared in the harvest bottle of the system. Although a small proportion of cells

appeared in the harvest of the other perfusion systems, the proportion was small enough to be negligible and did not affect the total densities determined in the bioreactor. This bleeding of cells into the harvest stream was also seen in the ESF experiments involving the HPCHO Chinese hamster ovary cells, but the bleeding of the hybridoma cells from the system was much less severe. The phenomenon appeared to be related to the rotational speed of the external spin filter module. The experiment began with an ESF rotational speed of 280 rpm, but many cells were being harvested rather than replaced into the bioreactor. The rotational speed of the external spin filter was increased to 300 rpm. At this point, the viability of the cells did not suffer due to the increased shear forces created by the increased rotational speed but a greater proportion of cells was retained in the bioreactor. Table 10 shows the two rotational speeds with the respective density found in the bioreactor, density found in the harvest bottle, and viability of the cells in the bioreactor.

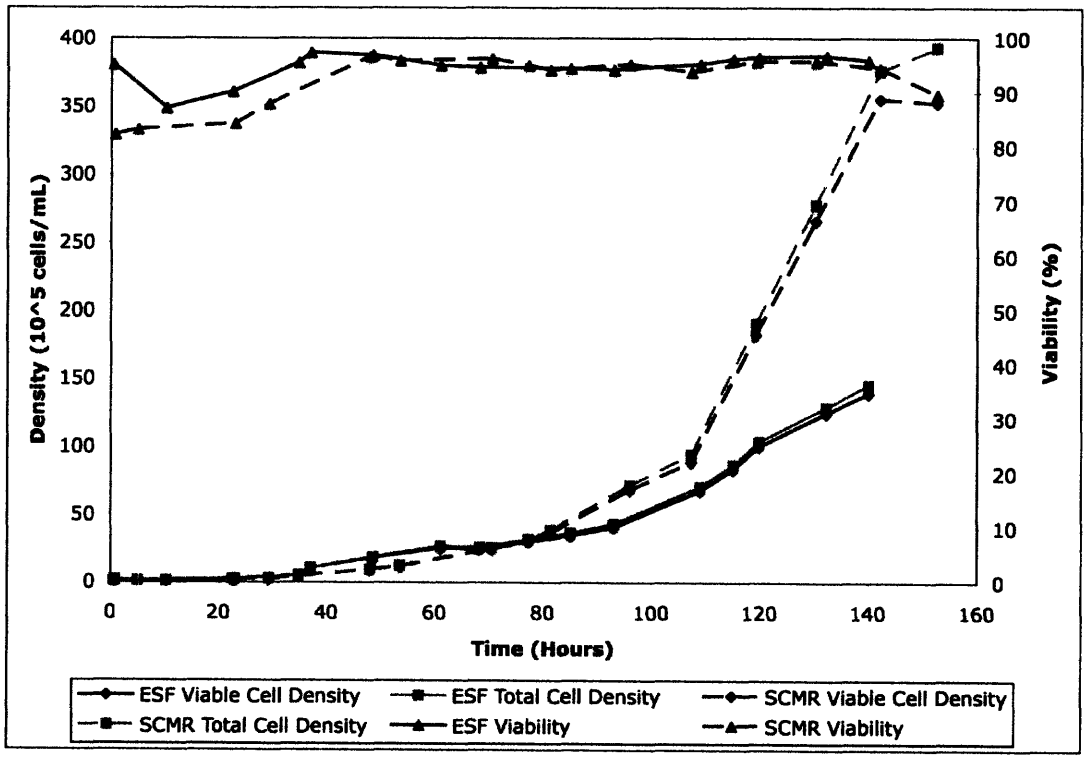

Figure 58 – Comparison of IB4 SCMR Run and IB4 ESF Run

As Figure 56 and Table 10 clearly illustrate, the external spin filter did not adversely affect the viability of the IB4 hybridoma cells. This was similar to the effect of the ceramic membrane module on the hybridoma cells and different from the effect of the

ATF hollow fiber membrane cartridge on the IB4 hybridoma cells. The viability of the IB4 culture immediately after inoculation was quite high. During the lag phase, the viability of the culture decreased below 90%. Feeding of fresh medium and harvesting of spent medium was initiated after 61 hours. By 35 hours, the viability of the ESF experiment was above 95% and remained above that value for the remainder of the experiment.

Table 10 – IB4 ESF Rotational Speeds and Corresponding Cell Densities in the Reactor and Harvest Bottle, and Cell Viability in the Reactor

ESF Rotational Speed	Total Density of Cells in Reactor	Total Density of Cells in Harvest	Viability of Cells in Reactor
280 rpm	26.05×10^5 cells/mL	3.77×10^5 cells/mL	95.1 %
300 rpm	128.83×10^5 cells/mL	6.11×10^5 cells/mL	96.8 %

Although cell viability did not become a major issue in the IB4 ESF experiment, cell aggregation and clumping did become a major issue. Cell aggregation was observed in the ATF and SCMR systems, but the cell aggregation in the IB4 ESF experiment was so extensive, the clumps of cells would congregate in the lines from the bioreactor to the spin filter apparatus and the lines from the spin filter apparatus back to the bioreactor. It was necessary to periodically flush the cell clumps from the lines. The problem was so extensive, that it halted the experiment after 140 hours when the clumps prevented further harvesting from the bioreactor. A possible solution to this problem, a modified external spin filter apparatus, was proposed and will be further discussed.

The concentrations of the major metabolites in the IB4 ESF experiment are shown in Figure 59. The concentration of glucose in the IB4 culture at the inoculation of the ESF experiment was about 5.6 g/L. After 10 hours, during which the viability of the culture decreased and the hybridoma cells adjusted to the new environment, the glucose concentration began decreasing steadily until it was about 3.9 g/L at 61 hours. Perfusion of the IB4 ESF system was begun at this time. After perfusion of fresh medium was initiated, the glucose concentration increased to about 4.25 g/L at 68 hours. After this point, the concentration of the major nutrient began decreasing steadily again until it reached a final value of about 1.5 g/L at 140 hours. Apparently, the perfusion rate of the system was not high enough to fully replenish the glucose in the system. Initially, there

was no lactate in the IB4 culture. After the lag phase ended at about 25 hours, the concentration of lactate began increasing. The lactate concentration was 1.3 g/L by 61 hours. This meant that a slightly smaller amount of lactate (1.3 g/L) was produced by the consumption of a slightly larger amount of glucose (1.7 g/L) by the hybridoma cells. It can be assumed that a small amount of IgG_{2a} monoclonal antibody was produced during the batch phase of this ESF experiment since the entirety of the glucose was not converted to lactate via cellular metabolism. Perfusion was initiated at this time. After 61 hours, the lactate concentration decreased slightly to less than 1.1 g/L. This showed that the external spin filter module was initially effective at removing the excess lactate from the IB4 culture. After 68 hours, the lactate concentration began increasing until it was 2.4 g/L at 132 hours. It is difficult to determine how much of the glucose consumed by the hybridoma cells during the perfusion phase of the experiment was utilized for the production of monoclonal antibody since the lactate was removed from the system. After 132 hours, the lactate concentration increased dramatically. If it is assumed that the lactate was removed from the system at the same rate that the glucose was supplied to the system, the fact that the lactate concentration increased at a slower rate than the glucose concentration decreased showed that some monoclonal antibody was produced during the perfusion phase of the experiment. The external spin filter was no longer effective at removing excess lactate from the system. In fact, the external spin filter was no longer effective in removing anything from the system due to the large amounts of cell aggregation present in the bioreactor and the lines of the spin filter module. The final concentration of lactate in the culture was 6.4 g/L at 140 hours.

The concentration of glutamine in the IB4 culture at the inoculation of the ESF experiment was almost 8 mmol/L. After 10 hours, during which the viability of the culture decreased and the hybridoma cells adjusted to the new environment, the glutamine concentration began decreasing steadily until it was 4.6 mmol/L at 61 hours. Perfusion was initiated at this time. Perfusion of fresh medium initially increased the glutamine concentration to over 5.1 mmol/L at 68 hours. After this time, the glutamine concentration began decreasing again, though at a slower rate than was seen in the batch phase of the experiment. Apparently, the perfusion rate was not high enough to fully replenish the glutamine concentration in the culture. When the experiment was halted

after 140 hours due to cell clumping, the glutamine concentration in the medium was about 3 mmol/L. Initially, the concentration of ammonium ion in the IB4 culture was 1.25 mmol/L. The ammonium ion concentration increased steadily after inoculation until it reached nearly 6.2 mmol/L at 61 hours. Feeding of fresh medium and harvesting of spent medium was begun at this time. During the perfusion phase of the experiment, the concentration of ammonium ion fluctuated, but the average value remained 6 mmol/L for the remainder of the experiment. This showed that the external spin filter module was effective at removing excess ammonium ion from the IB4 culture. Maintaining a relatively low concentration of this toxic metabolite allowed the hybridoma cells to continue taking up glucose for the purposes of cellular metabolism as well as monoclonal antibody production. Since the glutamine concentration continued to decrease during this time, however, it is not likely that a significant amount of IgG_{2a} monoclonal antibody was produced.

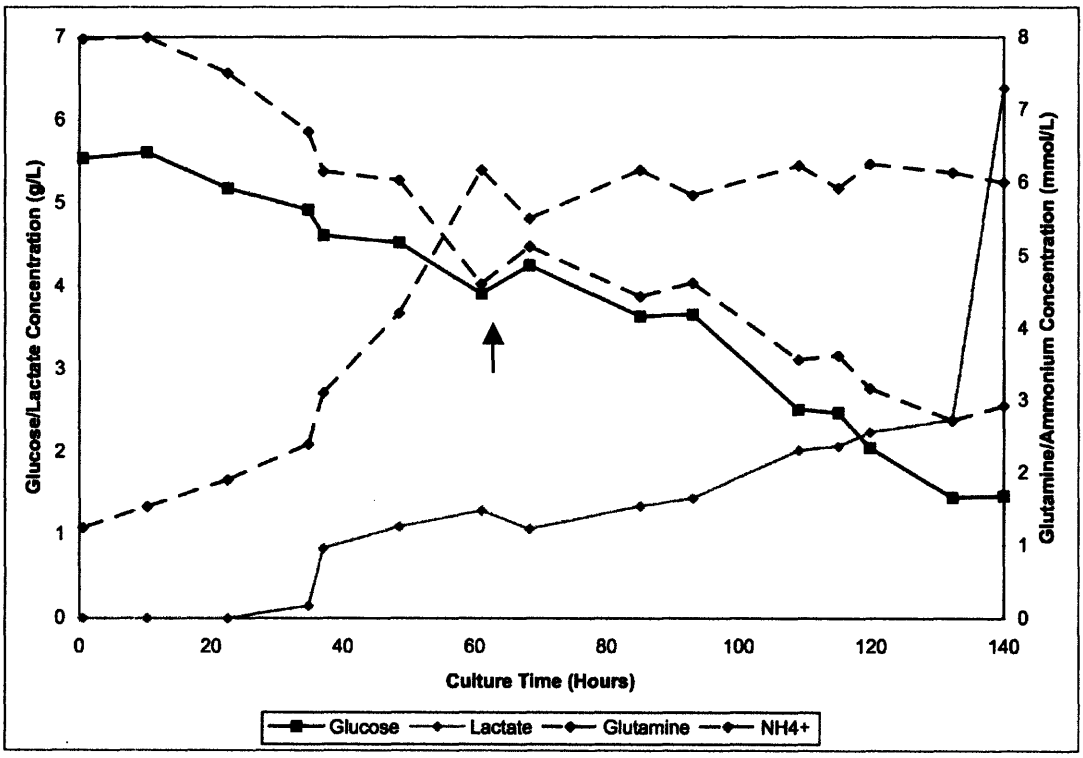

Figure 59 – Metabolic Data for IB4 ESF Run. The black arrow indicates the start of perfusion.

The IgG_{2a} monoclonal antibody concentration in the IB4 ESF experiment was determined periodically. The capillary electrophoresis data illustrating the product concentration of the hybridoma ESF culture over time is shown in Figure 60. There were

no detectable levels of antibody while the hybridoma cells were in the lag phase and the early stages of the exponential growth phase. The concentration of IgG_{2a} monoclonal antibody in the ESF culture increased during the later stages of the exponential growth phase. The monoclonal antibody concentration increased to over 50 ng/mL by 61 hours. Perfusion was initiated at this time. Because the external spin filter apparatus was effective at removing the product from the culture, the monoclonal antibody concentration had dropped to about 20 ng/mL by 68 hours. After this point, the monoclonal antibody concentration in the bioreactor varied widely, reaching a maximum value of 46 ng/mL and a minimum value of 0 ng/mL by the end of the experiment. The concentrations of monoclonal antibody found in the ESF reactor during this experiment were not significantly different than the product concentrations observed in the batch and fed-batch experiments. They were also much lower than the product concentrations observed in the ATF experiment and drastically lower than the product concentrations observed in the SCMR experiment.

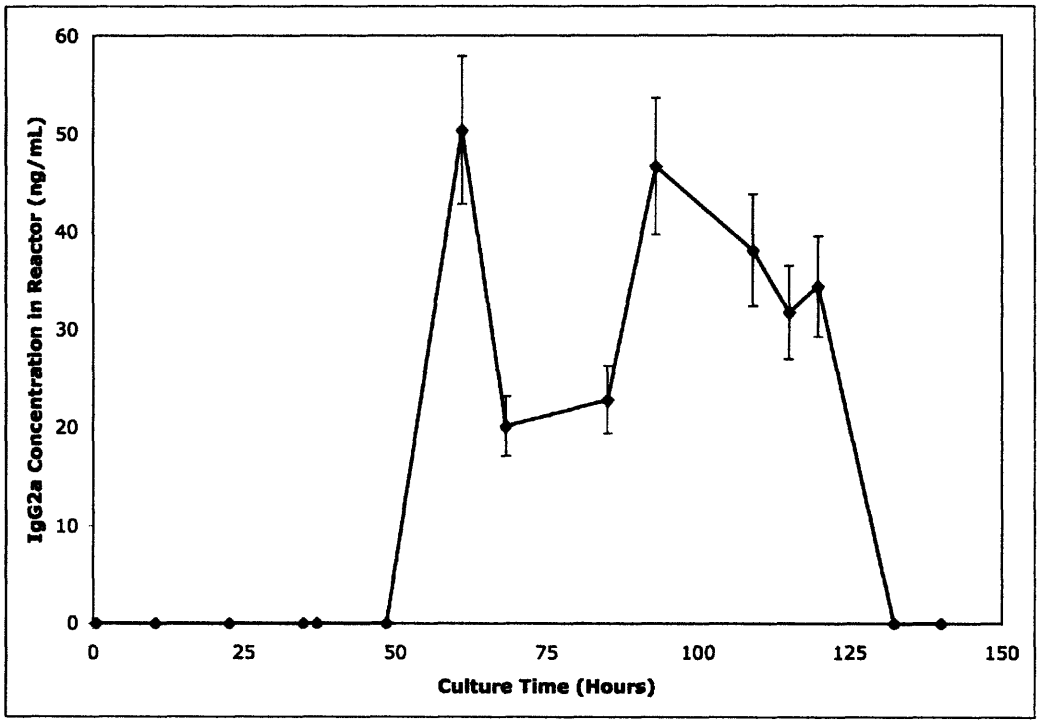

Figure 60 – Monoclonal Antibody Concentration Inside Reactor vs. Time for IB4 ESF Run. The error bars correspond to one standard deviation.

The total amount of monoclonal antibody produced by the IB4 ESF culture over time is shown in Figure 61. The qualitative trend illustrated by Figure 60 is evident but

slightly altered in Figure 61. Initially, there was no detectable amount of IgG_{2a} monoclonal antibody produced by the system. After 50 hours, a total amount of over 90 mg of monoclonal antibody was produced. After perfusion began at 61 hours, the amount of antibody produced decreased to 36 mg. This could have been due to error in the capillary electrophoresis measurement. The total amount of antibody produced by the ESF system gradually increased after this time to a final value of 175 mg. The total amount of antibody produced did not decrease at the end of the experiment because antibody was continually harvested from the system even though little antibody was present in the bioreactor itself. This characteristic was similar to the results found for the IB4 hybridoma experiments with the ATF system and allowed quicker access for downstream processing of the product. Much less IgG_{2a} monoclonal antibody was produced by the ESF system than either the SCMR or ATF systems.

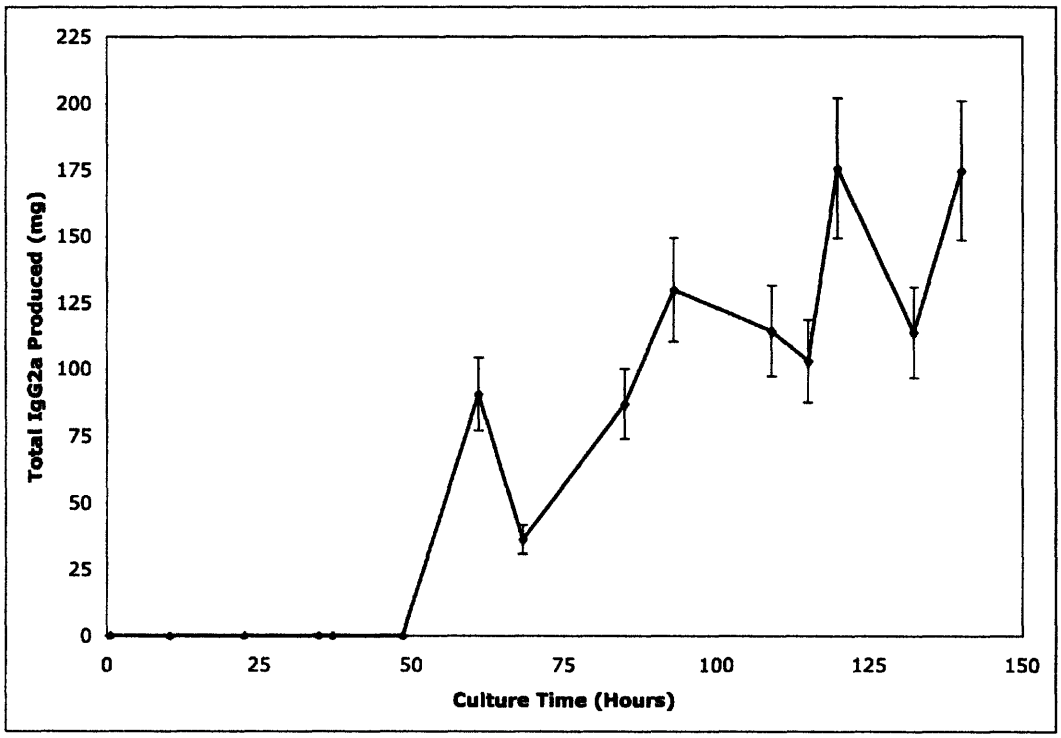

Figure 61 – Total Monoclonal Antibody Produced vs. Time for IB4 ESF Run. The error bars correspond to one standard deviation.

Because the results of the IB4 ESF experiments were more promising then the results of the HPCHO ESF experiments, another series of experiments were performed with the hybridoma cells. In this set of experiments, the external spin filter module was

modified. In the traditional set-up of the spin filter module, a steel rod sits in the center of the module, inside the steel mesh. This steel rod pulls medium that traverses the steel mesh from the bioreactor. In the modified spin filter module, several baffles were added to the steel rod in the center of the spin filter module. The baffles created turbulent flow at a lower rotational speed. It was hoped that the retention of cells in the reactor would improve with this modified equipment. It was also hoped that the increased turbulence created by the baffles would reduce the amount of cell aggregation and clumping in the system.

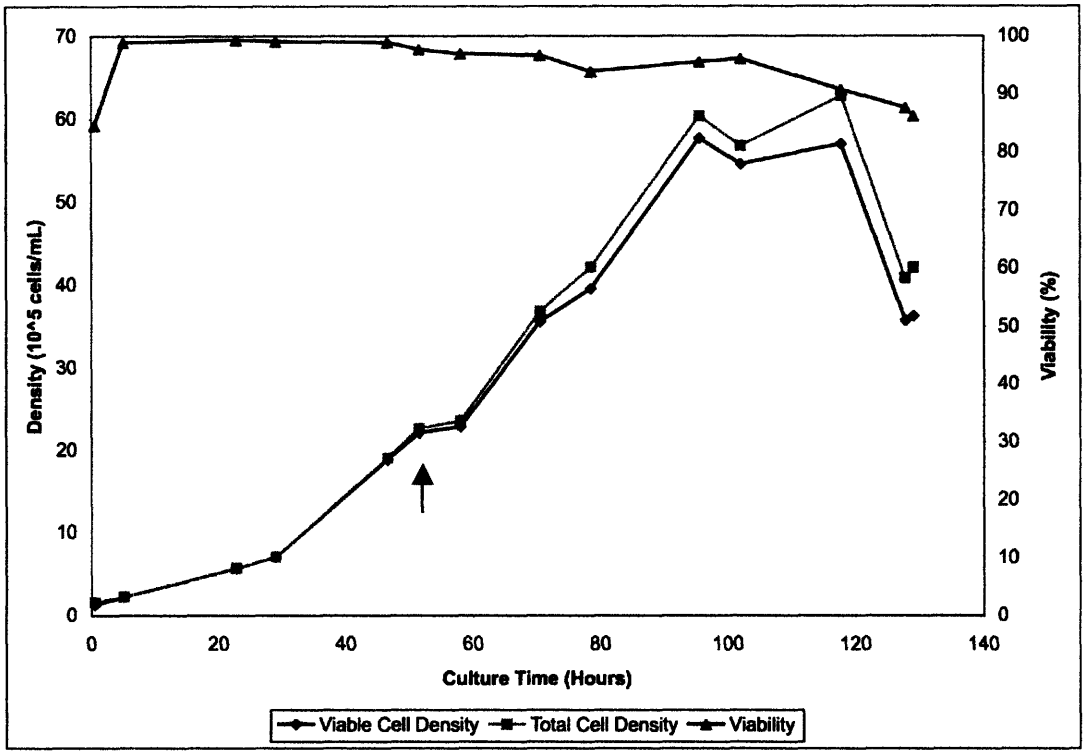

Figure 62 – Growth Data for IB4 Modified ESF Run. The black arrow indicates the start of perfusion.

The most successful modified ESF experiment completed with the IB4 hybridoma cell line will be discussed. The three-liter bioreactor was inoculated with a 1.8-liter working volume of IB4 culture with an initial density of around 1.5 x 10^5 cells/mL. Disturbances included the purging of the sample line of 3 mL and the sampling of 5 mL twice daily, as well as feeding of fresh medium and harvesting of spent medium after 53 hours. The autoclaved 3 liter bottles that contained the fresh medium to be fed and that collected the spent medium were replaced with a new set of autoclaved bottles twice,

after 78 and 102 hours. The total amount of medium supplied to the IB4 modified ESF experiment was 8.7 liters. The initial rotational speed of the external spin filter was 270 rpm, although this speed increased gradually through the course of the experiment. The final rotational speed of the external spin filter was 300 rpm. The recirculation rate began at 150 rpm, but was eventually increased to 200 rpm and then 225 rpm in an attempt to continue the experiment when cell clumping became noticeable. The perfusion rates were quite similar to those used in the previous ESF experiment shown in Table 9, though in the modified ESF experiment, perfusion was started 10 hours earlier and the experiment was halted 10 hours earlier.

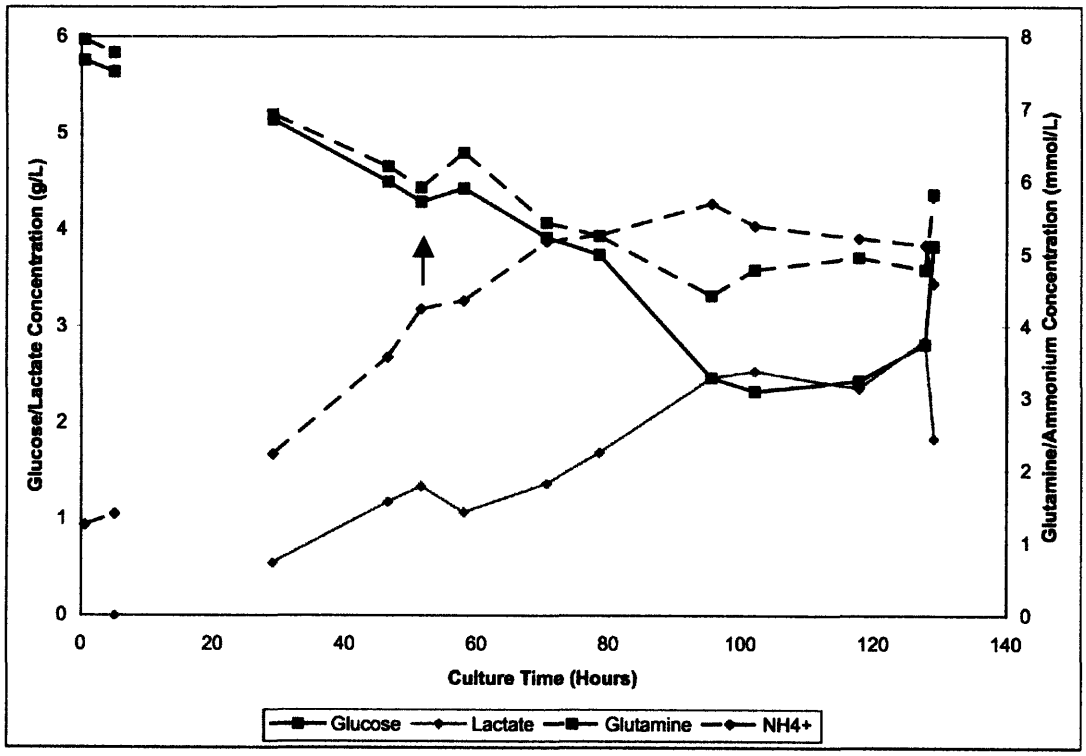

Figure 63 – Metabolic Data for IB4 Modified ESF Run. The black arrow indicates the start of perfusion.

The cell concentration and viability data for the IB4 modified ESF experiment is shown in Figure 62. The culture experienced a normal lag phase of about 25 hours. The exponential growth phase began at 25 hours and ended at about 95 hours. The culture experienced a stationary phase from 95 hours to 120 hours. After this point, a death phase was observed. The culture was halted at 130 hours due to fouling of the steel spin filter module by cell aggregates, which will be discussed further below. The

concentrations of the major metabolites in the modified ESF culture are shown in Figure 63. As these two figures show, the growth of the IB4 cells in the modified ESF experiment did not differ much from the growth of the IB4 cells in the batch and fed-batch experiment. Apparently, the baffles in the modified external spin filter apparatus did not improve the maximum viable cell concentration achieved by the experiment or alter the consumption of nutrients and production of waste products by the ESF system. In fact, the modified ESF experiment achieved the lowest maximum viable cell concentration of the four perfusion systems tested. The IgG_{2a} monoclonal antibody production of the modified ESF experiment was not analyzed by capillary electrophoresis because the cell concentration and viability results were disappointing.

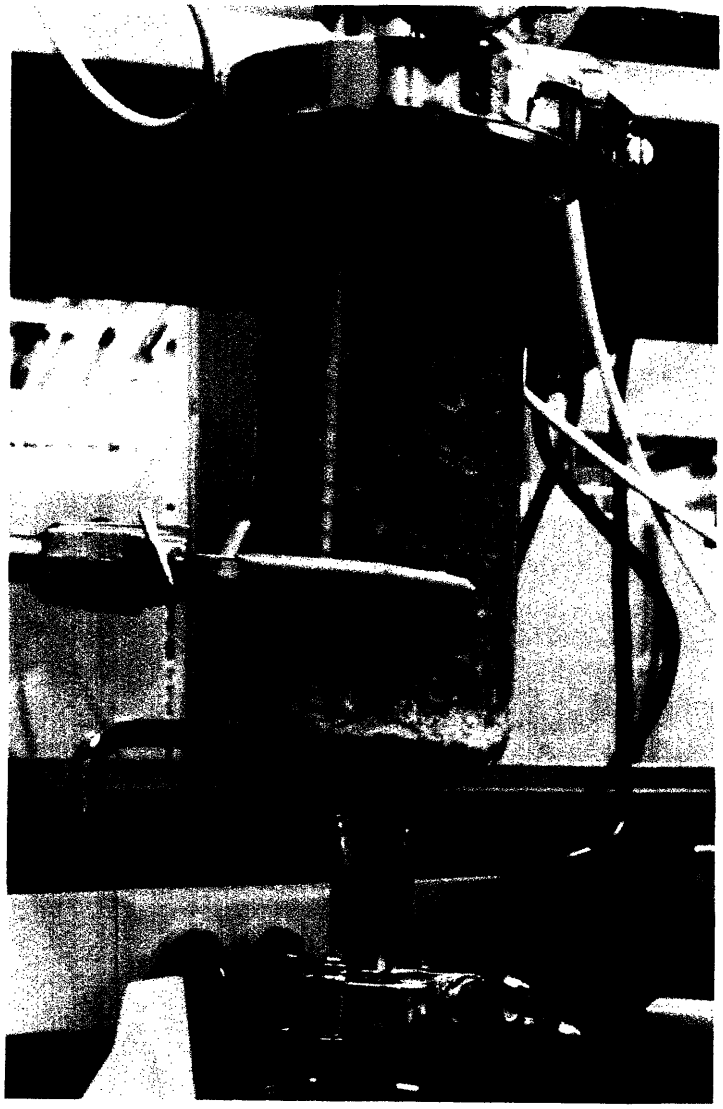

Figure 64 – Cell Aggregation and Clumping in IB4 Modified ESF Experiment

The baffles in the modified external spin filter apparatus did not significantly improve cell retention. In addition, the aggregation and clumping of IB4 hybridoma cells was actually made much worse by the use of the modified external spin filter. The digital photograph in Figure 64 of the modified spin filter apparatus towards the end of the experiment clearly shows large aggregates of IB4 hybridoma cells. The modified ESF experiment was halted after 130 hours due to the disruption of harvesting by the cell aggregates. Another modification may be needed to further improve the external spin filter system. Rather than adding baffles to the center rod, the external spin filter could be positioned horizontally, the connections to the bioreactor could be altered, or the peristaltic pump could be modified to help disrupt cell aggregates as the cell culture is harvested from the bioreactor.

5 Conclusions

5.1 Feeding Strategies of Mammalian Cells: Perfusion vs. Fed-Batch

The main objective of this research was to compare the growth and monoclonal antibody production by HPCHO Chinese hamster ovary cells and IB4 hybridoma cells in batch, fed-batch, and three perfusion systems: the stirred ceramic membrane reactor (SCMR), the alternating tangential flow (ATF) hollow fiber membrane system, and the external spin filter (ESF) system. This research was successful in developing a broad survey of process options for typical drug production vehicles in the biotechnology industry. The cell growth and productivity data, along with economic considerations, will be discussed here for the purpose of recommending the best feeding strategy for each of the two cell lines studied.

It was hypothesized that the fed-batch systems and the three perfusion systems would reach greater maximum cell concentrations and achieve greater monoclonal antibody productions than the corresponding batch systems for each cell line. This was observed in all of the perfusion experiments performed for each cell line. The perfusion systems reached the highest cell concentrations and produced the largest amounts of monoclonal antibodies. In the fed-batch experiments performed for each cell line, the maximum cell concentrations reached were either about the same as those for the batch systems or less than 20% greater than those for the batch systems. However, the fed-batch systems in fact exhibited greater productivity per cell then the batch systems as well as the perfusion systems.

To make a general recommendation of feeding strategy for the production of monoclonal antibodies by suspended mammalian cells from the research generated by this study, it is important to revisit Table 1. The direct comparison of batch, fed-batch, and perfusion feeding strategies provided by Table 1 is reproduced below in Table 11 with some variation. Information gathered from experimentation with the HPCHO Chinese hamster ovary and IB4 hybridoma cell lines have been incorporated into Table 11. Although the information provided in Table 1 showed that the drawbacks of perfusion may lead to the supremacy of fed-batch as a feeding strategy, the information

gathered from this research shows otherwise. The amount of additional material and time dedicated to the perfusion systems in this research was not great enough to reject this feeding strategy in favor of the fed-batch strategy.

Table 11 – Direct comparison of batch, fed-batch, and perfusion strategies (adapted from 1, 6, and research generated by this thesis).

Process characteristics	Batch	Fed-Batch	Spin-filter / Dialysis Membrane Perfusion
Cell generations	Low	Medium	Medium
Cell concentration	Low	Low - Medium	High
Volumetric productivity (reactor)	Low	Medium	High
Volumetric productivity (medium)	Medium	Medium - High	Low – Medium
Throughput (product/reactor)	Low	Low - Medium	High
Lot consistency	High	Unknown	Unknown
Cycle time	Short	Medium	Medium – Long
Turnaround time	Low	Medium	High
Waste generation	Low	Low	Medium
Required materials	Low	Medium	Medium – High
Operation	Simple	Simple	More complex
Failure risk	$\leq 5\%$	$\leq 5\%$	$\leq 10\%$
Process development	Low	Medium – High	High
Scale-up	Easy	Easy	More complex

The cell concentrations reached in the fed-batch experiments were not much higher than those achieved in the batch experiments. However, the perfusion experiments produced many more cells than either of the other two feeding strategies. The volumetric productivity of the perfusion systems, even when considering the amount of medium used, was still as high as that of the batch systems. The amount of product produced per cell was higher in the fed-batch system than in either of the other two systems, but the total monoclonal antibody production was hindered by the low cell concentration. It was not possible to evaluate the lot consistencies of the monoclonal antibodies produced by these experiments, so that issue will not be considered. The cycle time, turnaround time, required materials, and amount of waste produced by the perfusion experiments were all less than previously expected. Although the perfusion systems could have been bled of cells periodically to further extend the culture time of the experiment, this was not necessary to produce a significantly higher amount of

monoclonal antibody than was produced by the fed-batch experiments. Periodic cell bleeding also would have reduced the fouling of the various filters, therefore further increasing the amount of cells and monoclonal antibody produced by the systems. Finally, the process development of the fed-batch systems was much more complex than previously thought. Taken together, this information shows that from an economic standpoint, perfusion strategies are more worthwhile than fed-batch strategies for the cell lines studied. The perfusion systems generated more monoclonal antibody than any other system, but the additional material and time costs were not significant.

This thesis showed that perfusion was a better choice of feeding strategy than fed-batch for the production of monoclonal antibodies by suspended mammalian cells. The stirred ceramic membrane reactor (SCMR) proved to be the most valuable perfusion system for both cell lines, although it had been hypothesized that the external spin filter (ESF) system would outperform the SCMR. These conclusions may differ for various cell lines, so the results of the experiments for the HPCHO Chinese hamster ovary cells and the IB4 hybridoma cells will be revisited and discussed in context below. Much additional research is also needed and this future work will be discussed in the next section.

5.2 HPCHO Chinese Hamster Ovary Cells

Batch, fed-batch, and various perfusion experiments were performed with the HPCHO Chinese hamster ovary cells. The HPCHO cell line was chosen for this research because it is a typical example of a Chinese hamster ovary cell line that secretes monoclonal antibody as a product. The HPCHO experiments will be summarized briefly and a specific recommendation of feeding strategy for the production of IgG_1 monoclonal antibody by HPCHO cells will be made. Table 12 below summarizes the results of the five types of experiments performed with the HPCHO cells. The length of time of the experiment (culture time), the volume of medium consumed by the system during the experiment, the qualitative amount of IgG_1 monoclonal antibody produced, and the maximum cell concentrations achieved for the batch, fed-batch, SCMR, ATF, and ESF

experiments are listed in Table 12. A visual summary of the growth data from the various HPCHO experiments is shown in Figure 65.

The major drawback of the series of HPCHO experiments performed was that the IgG_1 monoclonal antibody concentration was not quantitatively analyzed. For this reason, the HPCHO data cannot be considered as in depth and accurate as the data presented from the IB4 experiments. Every effort was made, however, to qualitatively compare the productivity of each experiment by considering the maximum viable cell concentration achieved in the experiment, investigating the trends in metabolite consumption by the CHO cells, and noting the average cell diameter of the CHO cells and the osmolality of the medium through the course of the experiment.

Table 12 – Summary of Key Data for HPCHO Experiments

Feeding Strategy	Culture Time	Volume of Medium	Amount of IgG_1	Max. Viable Cell Density	Max. Total Cell Density
Batch	215 hours	1.80 L	Low	38.62×10^5 cells/mL	50.72×10^5 cells/mL
Fed-Batch 1	250 hours	2.65 L	Medium	31.37×10^5 cells/mL	36.13×10^5 cells/mL
Fed-Batch 2	145 hours	2.60 L	Medium	41.19×10^5 cells/mL	41.96×10^5 cells/mL
SCMR	175 hours	9.80 L	High	106.6×10^5 cells/mL	119.5×10^5 cells/mL
ATF	220 hours	19.6 L	High	107.16×10^5 cells/mL	176.67×10^5 cells/mL
ESF	165 hours	7.10 L	Medium	48.01×10^5 cells/mL	54.26×10^5 cells/mL

The batch HPCHO experiment lasted 250 hours, used 1.8 liters of medium, achieved moderate cell concentrations, but produced a low amount of IgG_1 monoclonal antibody. This was taken as the base case scenario for the HPCHO experiments. Two fed-batch HPCHO experiments were performed. The first fed-batch experiment was 250 hours long and produced more monoclonal antibody per cell, but fewer cells overall. The second fed-batch experiment was only 145 hours long and produced less monoclonal antibody per cell, but more cells overall. None of the fed-batch experiments performed with the HPCHO cells was successful in achieving a high cell concentration or a large amount of product.

It was determined that a bi-phasic fed-batch system would be the most effective fed-batch feeding strategy. Initially, enough medium would be supplied to allow the cells to reach a high concentration by the end of the batch phase. Next, a feed medium that would increase the osmolality of the system would be added. This increase in osmolality would cause stress to the HPCHO cells and cause them to enter a monoclonal antibody production phase.

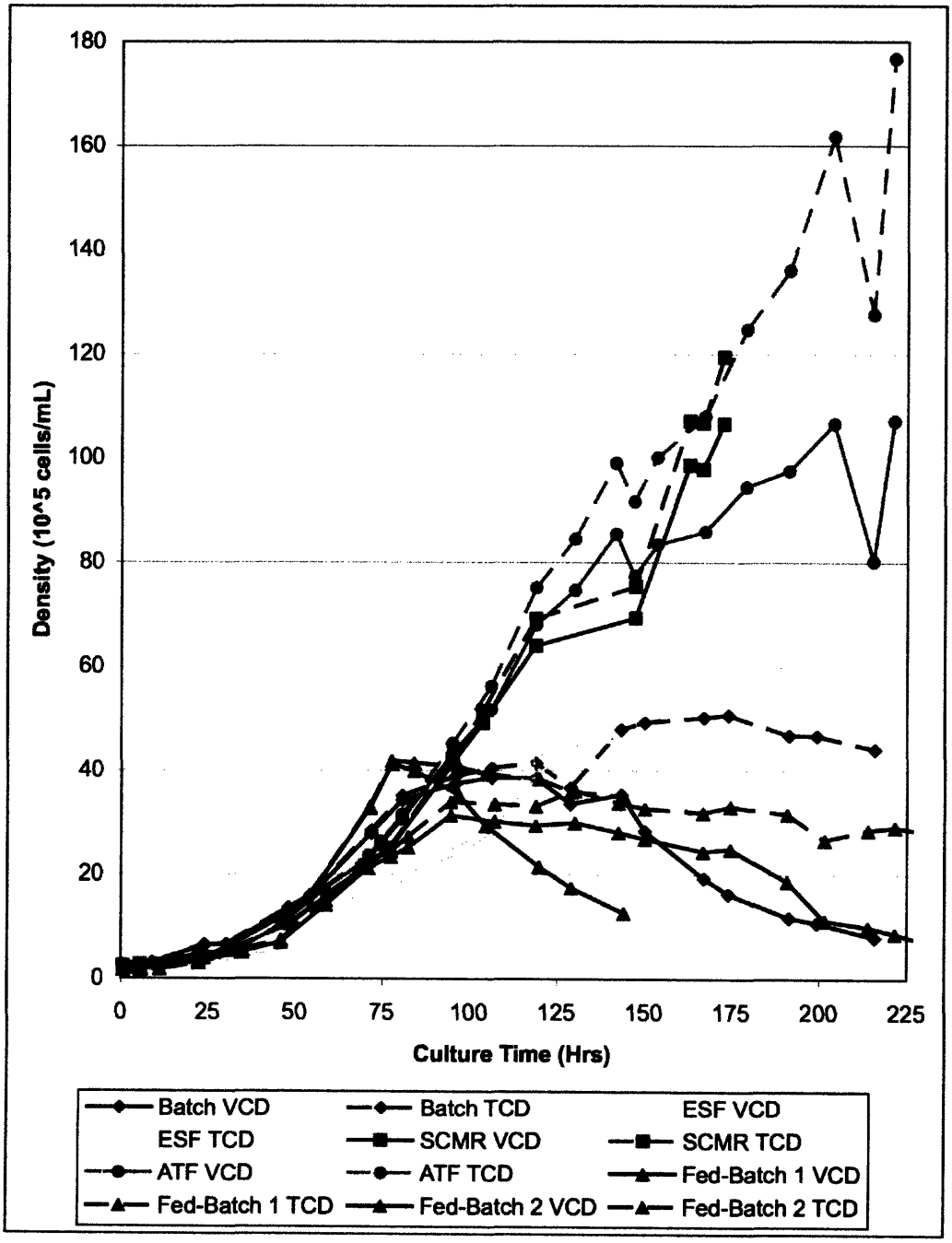

Figure 65 – Summary of Growth Data for HPCHO Experiments

The external spin filter experiment with the HPCHO cells was not very successful. A large proportion of CHO cells penetrated the spin filter and appeared in the harvest line. When the rotational speed of the ESF module was increased in an attempt to prevent the harvesting of cells, the viability of the culture was adversely affected. Therefore, the ESF system would not be a proper choice of feeding strategy for the HPCHO cells.

If cell growth is chosen as the basis of success, both the stirred ceramic membrane reactor and the alternating tangential flow hollow fiber membrane perfusion systems were quite successful feeding strategies. These experiments achieved similar maximum viable cell concentrations, although the ATF experiment reached a higher maximum total cell concentration. As a result, the viability of the HPCHO Chinese hamster ovary cells in the ATF experiment was not as high as in the SCMR experiment. If monoclonal antibody production is chosen as the basis of success, the SCMR and ATF perfusion systems again showed similar levels of success. Both systems produced the highest amounts of monoclonal antibody of all the HPCHO experiments performed. It is therefore necessary to compare the length of the two experiments as well as the amounts of materials used in the two experiments. The ATF experiment lasted 45 hours longer than the SCMR experiment. The ATF experiment also required nearly 10 more liters of medium than the SCMR experiment. There is no added benefit gained from utilizing more time and resources to run the ATF feeding strategy rather than the SCMR feeding strategy.

The experiments conducted with the HPCHO cells have shown that the proper choice in feeding strategy for producing monoclonal antibody from this cell line would be the SCMR system. This system achieved one of the highest HPCHO cell concentrations and produced one of the largest amounts of IgG_1 monoclonal antibody in the smallest amount of time and with the smallest amount of additional resources. The ceramic membrane module provided gentle filtration of the Chinese hamster ovary cells that maintained the high viability of the culture. The system could be further enhanced by periodic bleeding of cells and flushing of the filter to prevent fouling of the ceramic membrane module. The implications of scale-up of the SCMR system should also be investigated, but the stirred ceramic membrane reactor should be considered a viable

option for the production of monoclonal antibodies from other Chinese hamster ovary cell lines.

5.3 IB4 Hybridoma Cells

Batch, fed-batch, and various perfusion experiments were performed with the IB4 hybridoma cells. The IB4 cell line was chosen for this research because it is a typical example of a hybridoma cell line that secretes a monoclonal antibody as a product. The IB4 experiments will be summarized briefly and a specific recommendation of feeding strategy for the production of IgG_{2a} monoclonal antibody by IB4 hybridoma cells will be made. Table 13 below summarizes the results of the five types of experiments performed with the IB4 cells. The length of time of the experiment (culture time), the volume of medium consumed by the system during the experiment, the total quantitative amount of IgG_{2a} monoclonal antibody produced by the experiment, and the maximum cell concentrations achieved for the batch, fed-batch, SCMR, ATF, and ESF experiments are listed in Table 13.

Table 13 – Summary of Key Data for IB4 Experiments

Feeding Strategy	Culture Time	Volume of Medium	Amount of IgG_{2a}	Max. Viable Cell Density	Max. Total Cell Density
Batch	145 hours	1.80 L	130 mg	52.92×10^5 cells/mL	79.15×10^5 cells/mL
Fed-Batch	155 hours	2.65 L	160 mg	61.15×10^5 cells/mL	82.19×10^5 cells/mL
SCMR	155 hours	8.10 L	1320 mg	355.47×10^5 cells/mL	393.13×10^5 cells/mL
ATF	120 hours	7.40 L	570 mg	252.62×10^5 cells/mL	287.03×10^5 cells/mL
ESF	140 hours	9.60 L	175 mg	139.28×10^5 cells/mL	145.64×10^5 cells/mL

The major strength of the series of IB4 hybridoma experiments performed was that the IgG_{2a} monoclonal antibody concentration was quantitatively analyzed by capillary electrophoresis. For this reason, the IB4 data can be considered more in depth and accurate than the data presented from the HPCHO experiments. The IB4 monoclonal antibody production data can also be used to verify the qualitative results of the HPCHO

experiments. Although the two cell lines are not the same, they exhibit similar qualitative behavior.

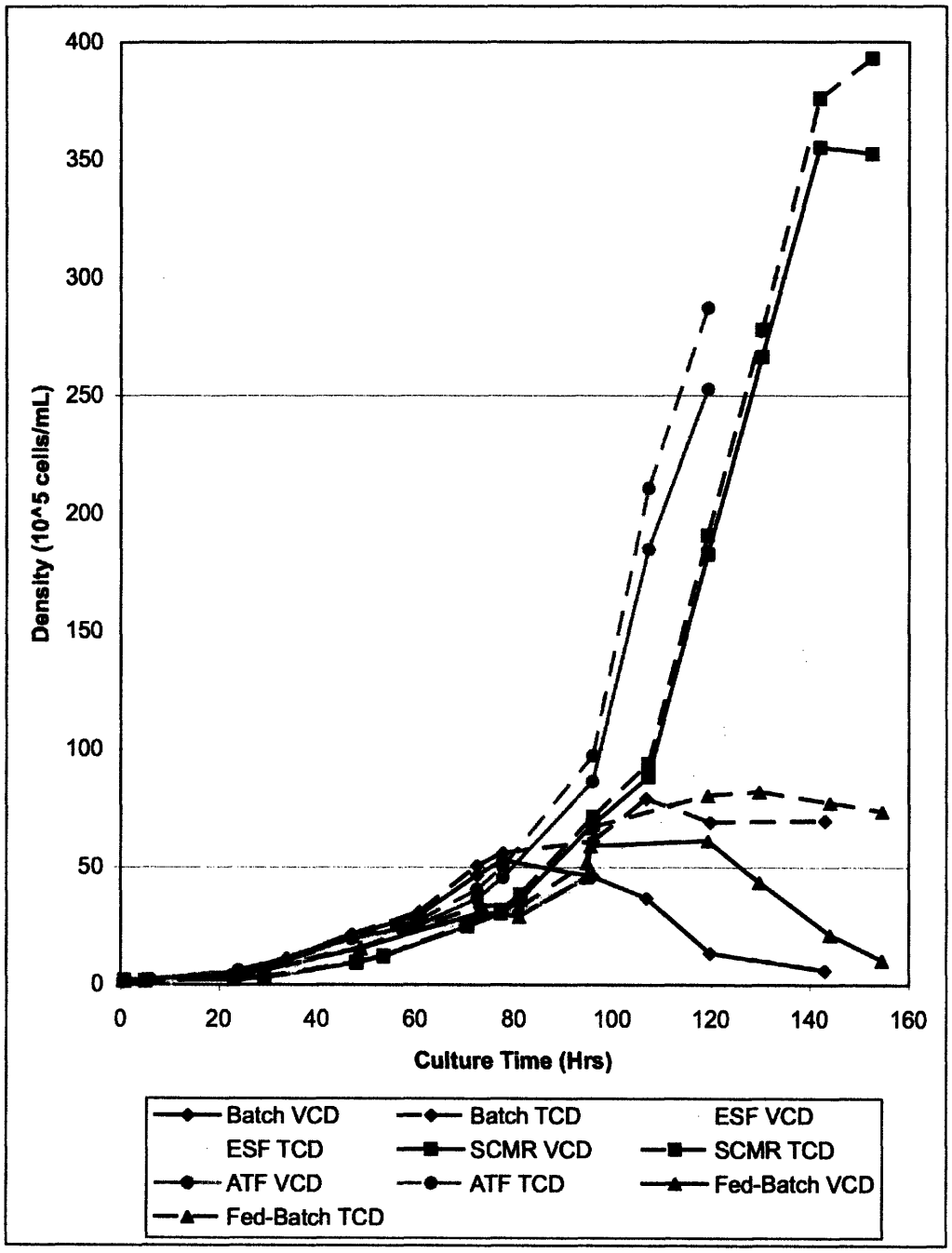

Figure 66 – Summary Growth Data for IB4 Experiments

The batch IB4 experiment lasted 145 hours, used 1.8 liters of medium, achieved moderate cell concentrations, but produced only 130 mg of IgG_{2a} monoclonal antibody. This was taken as the base case scenario for the IB4 experiments. The fed-batch IB4

experiment was 155 hours long, reached slightly higher cell concentrations, but produced only 160 mg of IgG_{2a} monoclonal antibody. The fed-batch experiments performed with the IB4 cells was not successful in achieving a high cell concentration and a large amount of product in comparison to the batch experiment. These quantitative results for the IB4 cells were quite similar to the qualitative results determined for the HPCHO cells. Either a much better fed-batch strategy must be found for the IB4 cells, or the fed-batch operation mode is not the proper choice for the production of monoclonal antibody by IB4 hybridoma cells.

The external spin filter experiment with the IB4 cells was more successful then the ESF experiment with the HPCHO cells, but it was not very successful overall. The cell concentrations achieved by the IB4 ESF system were nearly three times that of the batch system and over twice that of the fed-batch system. The greater number of cells, however, did not produce a significantly greater amount of monoclonal antibody. Only 175 mg of IgG_{2a} was produced by the ESF culture. A significant proportion of IB4 cells penetrated the spin filter and appeared in the harvest line. The rotational speed of the ESF module was varied throughout the run. Although increasing the rotational speed of the ESF module seemed to decrease the amount of hybridoma cells that were harvested without significantly affecting the viability of the culture, large aggregates of hybridoma cells began to appear. The perfusion experiment was eventually halted due to the aggregates clogging the lines of the system. Experiments were also performed with an external spin filter that had been modified by the addition of baffles. This modified ESF module did not improve the cell retention or aggregation problems experienced in the previous IB4 ESF experiments. As was the case with the HPCHO Chinese hamster ovary cells, the ESF system would not be a proper choice of feeding strategy for the IB4 hybridoma cells.

The alternating tangential flow hollow fiber membrane experiment with the IB4 cells was quite successful. The cell concentrations achieved by the IB4 ATF system were over five times that of the batch system and much greater than those achieved by the ESF system. Although the shear forces generated by the alternating tangential flow had a small adverse effect on the viability of the hybridoma cells, this effect was much smaller than that on the viability of the Chinese hamster ovary cells. The greater number of cells

led to the production of a significantly greater amount of monoclonal antibody. About 570 mg of IgG_{2a} was produced by the ATF culture in only 120 hours. This enhanced production was also achieved with a smaller amount of additional medium than was used in the ESF experiment. These quantitative results for the IB4 ATF system were similar to the qualitative results observed in the HPCHO experiments, except that the HPCHO ATF system had results that were much more similar to the results of the HPCHO SCMR system but the IB4 ATF system had results that were dissimilar to the results of the IB4 SCMR system. The main disadvantage of utilizing the ATF system when growing IB4 hybridoma cells was the need to halt the system due to clogging of the membrane cartridge by aggregated hybridoma cells. If the system had been allowed to continue to operate, it may have performed the best of all of the methods studied, but the hollow fiber membrane cartridge was particularly susceptible to fouling and the alternating tangential flow of the culture through the system was not enough to overcome this issue.

The stirred ceramic membrane reactor experiments with the IB4 hybridoma cells produced the best results of the perfusion systems tested. The cell concentrations achieved by the IB4 SCMR system were over seven times that of the batch system and much greater than those achieved by either the ESF or the ATF systems. The viability of the culture remained very high throughout the experiment. The very high concentration of cells led to the production of the largest amount of IgG_{2a} monoclonal antibody of any of the IB4 experiments: about 1.32 g. The experiment lasted 140 hours, about as long as the batch IB4 experiment lasted. A moderate amount of additional medium was needed, but this was well utilized because a large amount of product was harvested from the system. About the same amount of medium was used in the three types of perfusion experiments, but the SCMR experiment produced by far the most monoclonal antibody with that medium. Although the IB4 SCMR experiment was also halted due to aggregated cells fouling the ceramic membrane module, this occurred after a stationary phase was observed. It was possible to maximize the cell concentration and productivity of the IB4 SCMR system because the ceramic membrane module was less affected by aggregates of hybridoma cells.

As was observed in the HPCHO experiments, the experiments conducted with the IB4 cells have shown that the proper choice in feeding strategy for producing monoclonal

antibody from this cell line would be the SCMR system. This system achieved very impressive IB4 cell concentrations and produced by far the largest amounts of IgG_{2a} monoclonal antibody of all the IB4 experiments in a relatively short amount of time and with a relatively small amount of additional resources. The ceramic membrane module provided gentle filtration of the IB4 hybridoma cells that maintained the high viability of the culture. The SCMR system was also the least affected by aggregation of the hybridoma cells. The system could be further enhanced by periodic bleeding of cells and flushing of the filter to prevent fouling of the ceramic membrane module and any excessive aggregation of the IB4 hybridoma cells. The implications of scale-up of the SCMR system should also be investigated, but the stirred ceramic membrane reactor should be considered a viable option for the production of monoclonal antibodies from other hybridoma cell lines.

6 Future Directions

The work begun by this thesis could be continued in a variety of important ways. Three main categories of future work exist: the analysis of the concentrations of monoclonal antibodies produced by the HPCHO Chinese hamster ovary cells and the IB4 hybridoma cells, further optimization of the fed-batch feeding strategies for these two cell lines, and further experimentation with various perfusion strategies for these two cell lines.

6.1 Analysis of Monoclonal Antibodies

The first major area of future work regarding the research presented in this thesis involves the analysis of the monoclonal antibody production of the HPCHO Chinese hamster ovary cells and the IB4 hybridoma cells. Three main objectives must be fulfilled in this area. First, the production of the HPCHO cells must be further investigated. Second, a more accurate and precise method of quantification must be used to analyze monoclonal antibody content. Finally, detailed characteristics of the product, such as glycosylation, must be evaluated.

Although the production of IgG_1 by the HPCHO Chinese hamster ovary cells grown by various feeding strategies was qualitatively estimated by metabolite consumption, average cell diameter, and medium osmolality data in this thesis, the monoclonal antibody production was not evaluated quantitatively by the capillary electrophoresis equipment. The IgG_1 antibody produced by the CHO cells was not detectable by this instrument. This may be a fault of the instrument, in which case a better quantitative analysis method must be considered, as will be discussed below. This may also be due to low production by the CHO cells in general. The HPCHO line used in this study may have been previously changed in some manner that limited the monoclonal antibody production of the line. Thawing of old samples of the cell line for testing is needed. The medium supplementation of the cell line should also be considered. Different concentrations and combinations of insulin and methotrexate were considered during the early stages of this research, but little effect on the production of

the HPCHO cells was found. A more rigorous medium study is needed to amplify the IgG_1 production of these CHO cells.

Further evaluation of the capillary electrophoresis instrument used to quantify the IgG_{2a} monoclonal antibody produced by the IB4 hybridoma cells in this research is needed. Complex analysis was hindered by time and materials limitations, but the initial evaluation showed a variation of 15% between repeated samples. A more quantitative and reliable method may be necessary to carefully compare production of the cell lines utilized in this study. As stated previously, the instrument was not able to detect concentrations below 10 ng/mL and the data was nonlinear below concentrations of 25 ng/mL. In addition, the inability to quantify the production of IgG_1 monoclonal antibody by the HPCHO Chinese hamster ovary cells may have been a fault of the capillary electrophoresis instrument rather than a characteristic of the cells, as discussed above. High performance liquid chromatography (HPLC) may be a viable option.

Analyzing detailed characteristics of mammalian cell culture products is becoming increasingly more important in an industry driven by Food and Drug Administration (FDA) regulations. For this reason, it is necessary to evaluate the structures of the monoclonal antibody produced by the cell lines in this research. It has been shown that different feeding strategies and culture conditions can lead to differences in the glycoslyation of murine IgG monoclonal antibodies (33). This is a particularly important characteristic of products to evaluate because glycosylation of IgGs influences biological function and physicochemical properties of the antibodies (35-37). Since the IgGs produced by the two cell lines utilized in this study would eventually be administered *in vivo*, structurally defined and biochemical consistent products are desired by the FDA. Glycosylation of the products could be analyzed by capillary electrophoresis, gel electrophoresis, HPLC, or another form of very precise protein measurement.

6.2 **Optimized Fed-Batch System**

The second major area of future work regarding the research presented in this thesis involves optimization of the fed-batch feeding strategies for both the HPCHO Chinese

hamster ovary cells and the IB4 hybridoma cells. Two main aspects of the current fed-batch feeding strategies for these cells must be evaluated. First, a specialized supplemental medium must be formulated for each cell line. Second, further optimization of the timing and dosage of each stage of the fed-batch experiments must be performed.

In this research, concentrated forms of the medium used in the batch cultures of the suspended mammalian cells were utilized in the fed-batch cultures of the cells. This is not an ideal feeding strategy because the mammalian cells may require different nutrients and conditions during the fed-batch phase of the experiment than they did during the initial stages of the batch phase of the experiment. A specialized supplemental medium must be formulated for each cell line. From the experiments involving the HPCHO Chinese hamster ovary cells presented in this research, the fed-batch process for this cell line should be bi-phasic. The cells should be grown to a maximum density, and then medium components causing osmotic stress to the cells could be added to create a production phase. From the experiments involving the IB4 hybridoma cells presented in this research, the fed-batch medium for this cell line should contain glucose to allow the cells to continue to grow and produce antibody, but the minimal amount of glutamine necessary to allow the cells to continue to divide. More research is needed to determine other nutrients and components that should be added to each fed-batch process.

Once specialized fed-batch mediums are developed for the HPCHO and IB4 cell lines, the timing and dosage of the fed-batch processes should be optimized. The HPCHO Chinese hamster ovary cell system should probably involve a single dose that increases the osmolality of the system. However, more research is needed to determine if this would be the most effective strategy. The feeding of the IB4 hybridoma system could be much more complex and experimentation involving the initiation of feeding, the volumes of doses, and the number of feedings to this system is needed.

6.3 Alternative Perfusion Systems

The last major area of future work regarding the research presented in this thesis involves experimentation with other forms of perfusion for growing the HPCHO Chinese

hamster ovary cells and the IB4 hybridoma cells. Two main aspects of perfusion for these cells must be evaluated. First, the perfusion systems utilized in this study could be modified in some manner to improve the performance of the system. Second, perfusion systems not utilized in this study could be considered.

Each of the perfusion systems utilized in this study can be modified in several ways in the attempt to improve the growth and monoclonal antibody production of the suspended mammalian cells studied. The stirred ceramic membrane reactor system was quite successful in achieving high cell densities, viabilities, and productivities of both the HPCHO Chinese hamster ovary cells and the IB4 hybridoma cells. There is, however, room for improvement with this system. Scale-up of this system may be considerably complicated due to the nature of the filter material. Scale-up of the SCMR system should be further investigated to determine if this would be a major issue. The number of ceramic cylinders could be doubled from ten to twenty. There was plenty of room in the bioreactor to allow for an increase in size of the ceramic membrane module. An increased surface area could potentially alleviate the fouling that eventually ended both the HPCHO and the IB4 SCMR cultures. A method to flush the system could also be devised to solve the fouling problem. Finally, the system could be bled of cells once the culture has reached a high density, in order to prolong the perfusion culture and generate a larger amount of monoclonal antibody.

Similar methods could be applied to the alternating tangential flow hollow fiber membrane system. The number of hollow fibers in the ATF membrane could be increased, thus increasing the surface area of the membrane module. The system already incorporates flushing by alternating the flow through the hollow fiber cartridge, but the system could be bled of cells to prolong the culture and generate a larger amount of monoclonal antibody. Another way to modify the ATF system would be to develop a system that causes less shear stress to the cells. The current ATF system adversely affected the viability of both the HPCHO Chinese hamster ovary cells and the IB4 hybridoma cells. A system utilizing lower pressure differences and gentler filtration would vastly improve the performance of the ATF device.

A major modification was applied to the external spin filter system in this study. Baffles were added to the ESF module in an attempt to increase the turbulence of the

flow near the filter, improve the cell retention of the perfusion device, and decreased the aggregation of the IB4 hybridoma cells. The results of the modified ESF experiments were not favorable, however. There are several other ways in which the ESF system may be modified to improve the growth and monoclonal antibody production of the HPCHO Chinese hamster ovary cells and the IB4 hybridoma cells. The actual filter module was constructed of steel. A different material could be used to construct the filter. The pore size of the filter could also be decreased to ensure that fewer cells would be able to pass through the mesh. The ESF system could be placed in a horizontal position rather than a vertical position, to minimize the places in the system in which aggregated cells could block the flow of the culture.

Some success was achieved in perfusion culture of suspended mammalian cells in this study with the SCMR and ATF systems. Future work could investigate alternative perfusion systems, however. Continuous centrifugation could be a promising alternative (11). In this perfusion system, culture would be removed from the bioreactor, gently centrifuged, and the cells would be returned to the bioreactor while the spent medium and monoclonal antibody were harvested. The novel Wave bioreactor technology discussed previously includes a filter device that can be used for perfusion of the Wave system. The fouling of various filters caused by aggregated cells could hinder the performance of this perfusion system, however.

A final method of perfusion culture involves the use of an acoustic filter. A BioSep system from Applikon Biotechnology (18-19) can be mounted directly on top of a bioreactor. A picture of this system is shown in Figure 67. Fresh medium may be continuously added to the reactor while spent medium is harvested from the BioSep chamber. Inside the chamber, cells are retained by ultrasonic forces and then washed back into the reactor. Medium is driven upwards through the chamber while an acoustic field is applied and a plane standing-wave generated. The ultrasonic forces produced drive the cells to the pressure nodes of the resonance field (18). The cells then travel laterally within the nodes to form aggregates that remain in the chamber. The separation of live cells from dead cells, debris, and medium is a result in differences in density, size, and compressibility (19). As the clumps grow larger, the cells are forced back down into the reactor where the clumps of cells are separated by the reactor's impeller. A great

advantage offered by acoustic filtration systems is that they can be adjusted to selectively retain viable cells while flushing out spent medium and cell debris. Since this separation is induced by ultrasonic standing waves, there is less concern about filter fouling than with other retention devices.

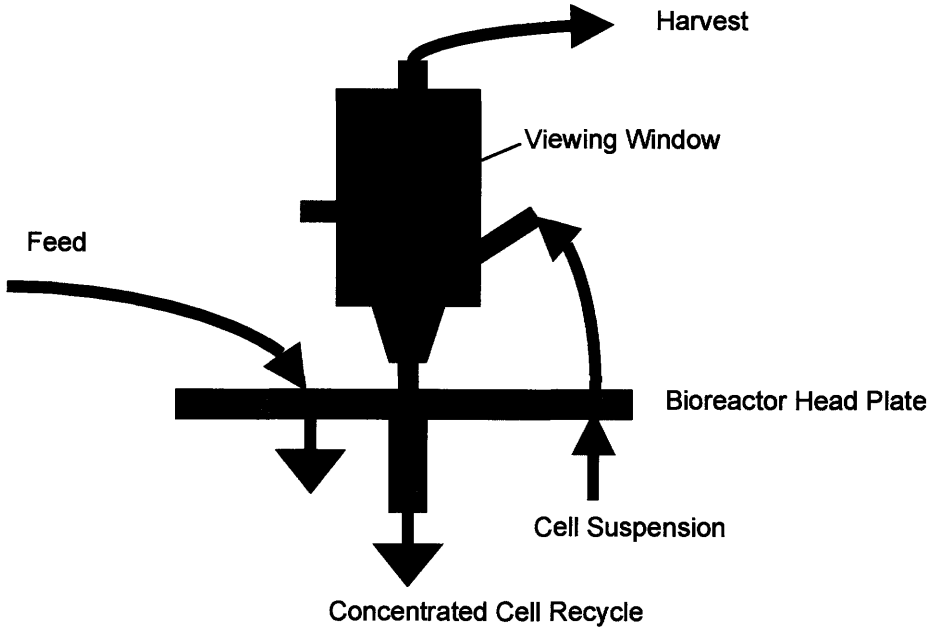

Figure 67 -- The Acoustic Filter System (adapted from schematic by Alyse Wu).

This thesis attempted to survey the growth and monoclonal antibody production of two types of suspended mammalian cells in batch, fed-batch, and perfusion operations. The research presented is an excellent starting point for future work. The quantification of monoclonal antibody produced by the HPCHO Chinese hamster ovary cells and the IB4 hybridoma cells should be further investigated. The optimization of the fed-batch feeding strategies for the two cell lines should be pursued. Finally, the perfusion systems studied in this thesis could be improved by modification and experimentation with other perfusion systems could be conducted.

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